UNIVERSITY OF ALBERTA

# MOLECULAR MECHANISMS UNDERLYING NORADRENERGIC MODULATION OF LONG-TERM HIPPOCAMPAL SYNAPTIC PLASTICITY

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Doctor of Philosophy*

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## Abstract

The noradrenergic neuromodulatory system widely innervates the mammalian brain, and impacts cognitive functions such as arousal, perception, learning, and memory. However, the cellular mechanisms that underlie the effects of noradrenaline (NA) on these higher cognitive processes are unclear. The persistent modification of synaptic strength, also known as synaptic plasticity, is a key candidate mechanism for some forms of memory in the hippocampus. Release of NA from noradrenergic afferents in the hippocampus strongly influences synaptic plasticity. As such, determining how NA modulates synaptic plasticity may provide crucial insights into the physiology of memory.

Here, a combination of *in vitro* electrophysiological and biochemical techniques were used to investigate the mechanistic role of NA in the long-term potentiation (LTP) of synaptic strength in the hippocampus. NA, acting through β-adrenergic receptors, was found to facilitate the induction and maintenance of LTP. Pairing β-adrenergic receptor activation with various patterns of electrical stimulation generated long-lasting LTP that was resistant to depotentiation, dependent on protein synthesis, and independent of transcription.

This β-adrenergic receptor-dependent LTP was stabilized by ERK- and mTOR-mediated activation of translation initiation machinery. Inhibition of PKA activity did not block the maintenance of β-adrenergic receptor-dependent LTP, or the engagement of protein synthesis in response to activation of β-adrenergic receptors. Moreover, activation of β-adrenergic receptors rescued deficient late-phase LTP induced by multiple trains of high-frequency electrical stimulation when PKA signaling was deficient. β-

adrenergic receptors therefore play a crucial role in gating the induction of long-lasting synaptic plasticity at the level of translation initiation via a PKA-independent pathway. Stimulation of the cAMP-dependent, but PKA-independent, Epac pathway also facilitated the maintenance of LTP via local translational regulation, suggesting the involvement of this signaling pathway in hippocampal synaptic plasticity.

Determining how NA influences information processing at cellular levels is essential for understanding memory processes. This thesis highlights the importance of noradrenergic receptors in regulating the induction and maintenance of synaptic plasticity in the hippocampus. These findings also provide evidence for a cellular mechanism that contributes to this regulation, and highlight the complex interplay of signal transduction pathways that are thought to underlie the formation and storage of memory.

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## List of Abbreviations

| | |
|---|---|
| 4E-BP | eIF4E binding protein |
| 5-HT | 5-hydroxytryptamine (serotonin) |
| 8-pCPT | 8-(4-chlorophenylthio)-2'-O-methyl-cAMP |
| A | adrenaline |
| AC | adenylyl cyclase |
| ACh | acetylcholine |
| ACSF | artificial cerebrospinal fluid |
| ACT-D | actinomycin D |
| AMPAR | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor |
| ANOVA | analysis of variance |
| AR | adrenergic receptor |
| BCM theory | Bienenstock, Cooper, Munro theory |
| BDNF | brain-derived neurotrophic factor |
| CA | *cornu Ammonis* |
| $Ca^{2+}$ | calcium |
| CaMKII | $Ca^{2+}$/calmodulin-dependent protein kinase II |
| cAMP | adenosine 3', 5'-cyclic monophosphate |
| cAMP-GEF | cAMP guanine exchange factor |
| CCAC | Canadian Council on Animal Care |
| CNS | central nervous system |
| COMT | catechol-O-methyltransferase |
| CREB | cAMP response element binding protein |
| CS | conditioned stimulus |
| DA | dopamine |
| DAG | diacylglycerol |
| DG | dentate gyrus |
| DMSO | dimethylsulfoxide |
| DPT | depotentiation |
| E-LTP | early phase LTP |
| EC | entorhinal cortex |
| ECL | enhanced chemiluminescence |
| eIF4E | eukaryotic translation initiation factor 4E |
| eIF4G | eukaryotic translation initiation factor 4G |
| Epac | exchange protein activated by cAMP |
| ERK | extracellular-signal regulated kinase |
| fEPSP | field extracellular postsynaptic potential |
| FMRP | fragile X mental retardation protein |
| G-protein | guanine nucleotide-binding regulatory protein |
| GABA | γ-aminobutyric acid |
| Glu | glutamate |
| GluR | glutamate receptor |
| HB | homogenization buffer |
| HFS | high frequency stimulation |
| IACUC | Institutional Animal Care and Use Committee |

| | |
|---|---|
| IEG | immediate early gene |
| I/O | input/output |
| IP3 | inositol triphosphate |
| ISO | isoproterenol |
| ITF | intermediate term facilitation |
| L-LTD | late phase LTD |
| L-LTP | late phase LTP |
| LA | lateral amygdala |
| LC | locus coeruleus |
| LFS | low frequency stimulation |
| LTD | long-term depression |
| LTM | long-term memory |
| LTP | long-term potentiation |
| MAO | monoamine oxidase |
| MAPK | mitogen-activated protein kinase |
| $Mg^{2+}$ | magnesium |
| mGluR | metabotropic glutamate receptor |
| MHPG | 3-methoxy-4-hydroxy-phenylethyleneglycol |
| Mnk1 | MAP kinase signal-integrating kinase |
| mRNA | messenger ribonucleic acid |
| MTL | medial temporal lobe |
| mTOR | mammalian target of rapamycin |
| NA | noradrenaline |
| NAT | noradrenaline transporter |
| NIH | National Institutes of Health |
| NM | normetanephrine |
| NMDAR | N-methyl-D-aspartate receptor |
| NR | neuromodulatory receptor |
| NST | nucleus of the solitary tract |
| NT-3 | neurotrophin 3 |
| OA | okadaic acid |
| PBS | phosphate buffered saline |
| PI3K | phosphoinositide 3-kinase |
| PKA | cAMP-dependent protein kinase |
| PKC | protein kinase C |
| PP1 | protein phosphatase 1 |
| PPF | paired pulse facilitation |
| PVDF | polyvinylidene difluoride |
| R(AB) | dominant negative inhibitor of PKA |
| Rap | rapamycin |
| Rp-cAMPs | adenosine 3', 5'-cyclic monophosphorothioate, Rp-isomer |
| SDS | sodium dodecyl sulfate |
| SEM | standard error |
| STM | short-term memory |
| SUB | subiculum |
| US | unconditioned stimulus |
| VDCC | voltage-dependent calcium channel |

# *CHAPTER I

## General Introduction

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## 1. Overview

The nature of memory has long been considered a subject that falls within the realm of philosophy rather than biology. Although the ancient philosopher and physician Hippocrates was convinced that thoughts, feelings, and perceptions originated from the brain, the profound complexity of the central nervous system greatly hampered experimental study for many centuries. The advent of advanced technology and enhanced understanding of natural phenomena has only recently placed questions concerning higher cognitive processes such as learning and memory squarely in the purview of biological analysis.

From the molecular biology of a single neuron to behavioural memory in an animal, numerous experimental approaches are currently employed to shed light on fundamental questions of memory: How do neurons store memory? Where is memory stored in the brain? What neural processes determine the strength and persistence of a memory? Substantial progress has been made toward addressing these questions, although our present understanding is at best incomplete.

Investigating the function of neurons on a cellular level, including examination of receptors, signaling molecules, and proteins, is a promising approach to enhance this understanding. Such a methodology adds definition to the information processing capacity of neurons while bypassing the inherent structural and behavioural complexity of the brain. A principal aim of this thesis is to examine the molecular and cellular mechanisms thought to contribute to the stability and potency of memory. Combining this approach with functional analysis of entire neural systems could lead to important insights into how neural networks generate long-lasting memory.

## 2. Learning and Memory

> "Memory is a gift of nature, the ability of living organisms to retain and to utilize acquired information or knowledge…Owners of biological memory systems are capable of behaving more appropriately at a later time because of their experiences at an earlier time, a feat not possible for organisms without memory." (Tulving, 1995)

The ability to acquire and retain behaviourally relevant information confers indisputable survival advantages. Organisms that can learn (gain information from the environment) and remember (maintain information over time) behave more efficiently and adaptively than organisms that are unable to utilize this information for decision-making (Klein et al., 2002). Retrieval of memory at a later time point provides an index of learning, and allows for persistent behavioural modification.

Logically, the functional organization of a memory system should reflect the design features necessary to solve recurrent evolutionary challenges. Appropriate decision-making often requires different types of information, including reflex patterns, representations, and associations. Furthermore, particularly salient information should be placed in long-term storage, whereas less significant information relevant only to the current situation can be quickly forgotten. These observations suggest that memory can be classified according to at least two separate parameters: the qualitative nature of the information stored, and the duration of retention.

### a) Multiple Memory Systems

As early as 1804, the philosopher Maine de Biran proposed that memory could be separated into three forms, each with distinct properties and mechanisms (Schacter and Tulving, 1994). He called these forms mechanical, representative, and sensitive memory. Although largely ignored at the time, de Biran's classification of memory was strikingly

prescient. Modern theories on the subject have renamed these systems procedural, declarative, and emotional memory, respectively (Schacter, 1990; Scoville and Milner, 1957; Squire, 1992). Procedural memory is expressed through performance, and allows for the gradual acquisition of patterns, habits, and skills. Conversely, declarative memory involves the conscious recollection of facts and events, and creates flexible representations. Memories of emotionally arousing experiences are modified by the emotional memory system.

Persuasive experimental evidence supporting the notion of multiple memory systems was first obtained from studies conducted on the amnesic patient H.M.. After surgery intended to remove epileptogenic tissue from the medial temporal lobe (MTL) and ameliorate H.M.'s intractable epilepsy, he developed severe anterograde amnesia. H.M. cannot effectively form new memories about people, places, facts or events, although his intellectual function is otherwise normal (Corkin, 1984; Milner et al., 1968; Scoville and Milner, 1957). In fact, he scores zero on tests designed to assess retention of pictures, short stories, lists of words and many other types of information. However, H.M. does retain specific types of memory (Corkin, 2002). One remarkable example of this spared capacity is H.M.'s ability to learn new sensorimotor skills. When assigned the task of 'mirror-drawing' (tracing a line drawing when only allowed to view both the drawing and one's hand through a mirror), H.M. demonstrated significant improvement during training trials, and retained the memory for this skill across training trials, despite an inability to remember having previously performed the task. H.M. also performs normally in repetition priming, classical conditioning, and habit learning. Taken

together, this pattern of memory deficits and capacities reflects a specific disruption of declarative memory, with procedural memory remaining intact.

A selective memory deficiency induced by surgical removal of brain tissue suggests that memory systems are functionally localized in the brain. Studies of memory function in other amnesiac patients also supported the notion of multiple, localized memory systems (Cohen and Squire, 1980; Squire and McKee, 1993; Tulving and Schacter, 1990; Warrington and Weiskrantz, 1968). However, in-depth understanding of anatomical structures involved in memory can only be examined in animal models that permit surgical and pharmacological manipulations of the brain. To this end, numerous animal models of amnesia were developed. Because rodents display an aptitude for various behavioural memory tasks, including conditioning, discrimination, and maze learning, this model was enthusiastically pursued. Despite apparently inconsistent initial results, studies of memory in rats and mice have provided insights into the nature and interactions of memory systems in the brain.

Parallel operation of memory strategies in rats was demonstrated using localized brain lesions. The anatomical location of the lesion differentially affected the performance of the rats on various memory tasks (Packard et al., 1989). A compelling example of multiple memory systems operating in intact animals involved training rats to navigate in a four-arm, cross-shaped maze (Packard and McGaugh, 1996). Rats were placed consistently in the south arm of the maze (with the north arm blocked) and trained to locate a reward in the west arm. A probe trial was then introduced wherein the rats were placed in the north arm of the maze (with the south arm blocked) and allowed to search for the reward. During probe trials administered early in training, rats entered the

west arm of the maze. This response indicated that the rats apparently remembered the 'place' (west arm) where the reward had been found previously. However, during probe trials administered later in training, rats entered the east arm of the maze. The rats therefore changed their memory strategy, instead remembering the 'response' (make a left turn from the starting position). The 'place' strategy is thought to reflect a factual, or declarative memory for the task, whereas the 'response' strategy represents a habit, or procedural memory for the same task.

Packard and McGaugh (1996) went on to demonstrate that the different memory strategies used by the rats were in fact mediated by different regions of the brain. Inactivation of specific brain regions was accomplished with localized lidocaine injections. Lidocaine injected into the hippocampus elicited random searching behaviour during the early probe trials, but did not affect the 'response' strategy exhibited during later probe trials. Conversely, lidocaine injected into the caudate nucleus did not disrupt the 'place' strategy used during early probe trials, but eliminated the 'response' strategy of later probe trials. Interestingly, inactivation of the caudate nucleus during later probe trials resulted in the rats resorting back to their original 'place' response to locate the reward. Taken together with results obtained from amnesiac patients and brain-lesioned animals, this double dissociation study suggests that the hippocampus and caudate nucleus are responsible for expressing distinct memory strategies that are generally consistent with declarative and procedural memory categorizations, respectively. Furthermore, hippocampus-dependent learning is acquired more quickly than caudate-dependent learning in this memory task, although both forms of learning are retained over

time. Separate brain regions therefore support different memory systems, and these systems can operate in parallel to generate behaviour (**Figure 1.1**).

Multiple memory systems also interact. The emotional memory system, which is activated in response to arousal, can influence performance on retention tasks dependent upon other memory systems. The amygdala is a brain region located in the medial temporal lobe that regulates autonomic, endocrine, motor and memory systems involved with emotional expression. Removal of the amygdala in monkeys leads to taming of aggression, and abnormalities of social behaviour (Kluver and Blucy, 1937). Similarly, human patients diagnosed with Urbach-Wiethe disease demonstrate bilateral calcification of the amygdala and consequent deficiencies in emotional processing (Markowitsch et al., 1994; Siebert et al., 2003).

Stimulation of the amygdala affects performance on both declarative and procedural memory tasks (McGaugh et al., 1996). Injection of amphetamine into the hippocampus or caudate nucleus facilitates retention for hippocampus- and caudate-dependent memory tasks, respectively. Amphetamine injected into the amygdala enhances performance on both hippocampus- and caudate-dependent memory tasks, although the amygdala is not necessary for subsequent retrieval of these memories. Therefore, activation of the emotional memory system can modulate memory formation and retention in other brain regions, highlighting the importance of interactions between multiple memory systems in the brain.

b) Memory Phases

Numerous classification systems have been employed to describe the variable duration of memories. Biochemical and neuropsychological data suggest that at least

three distinct phases of memory exist: working memory, short-tem memory (STM) and long-term memory (LTM). Working memory is proposed to last 10-30 seconds, and provides limited capacity information storage (Craik, 1979). Multiple components are thought to contribute to working memory, including a visuo-spatial sketchpad to hold and manipulate visual images, a phonological loop to retain speech-based information, an episodic buffer to integrate information into episodic events, and a central executive to act as attentional controller (Baddeley, 1996; Repovs and Baddeley, 2006). Cellular mechanisms in the prefrontal cortex play a crucial role in regulating working memory processes (Fuster, 1998; Goldman-Rakic, 1996). The separation between working memory and other phases of memory is illustrated by studies of human patients with selective damage to the MTL. As mentioned previously, these patients demonstrate severe anterograde amnesia. However, their capacity for immediate memory remains intact (Squire and Zola, 1997). H.M. is able to perform normally on tests of recognition and recall, as long as the task does not require retention after a significant delay, or involve distractions (Corkin, 2002).

STM permits memory retention for longer periods of time. Depending on the organism, STM can persist for minutes to hours. This phase of memory was originally postulated to be a precursor for LTM (James, 1890). There is also evidence to suggest that STM operates in parallel with LTM, rather than functioning as a step in the temporal progression of memory storage (Izquierdo and McGaugh, 2000). For example, injection of certain kinase inhibitors into a subregion of the hippocampus inhibits STM, without preventing the subsequent expression of LTM at a later time point (Izquierdo et al., 1999). Therefore STM may act as a temporary memory store that allows access to

information while slower neural processes operate separately, and in parallel, to establish the information in LTM.

The formation of LTM involves a process termed memory consolidation (McGaugh, 2000). Although regions dedicated to memory processing exist in the brain, the long-term repository for a memory is thought to be the neocortical area originally responsible for processing the information that comprises the memory (Alvarez and Squire, 1994; Bontempi et al., 1999). As such, consolidation is the slow, incremental reorganization of cortical circuits that permits permanent retention of memory. The time course of consolidation in humans is reflected in the phenomenon of temporally graded retrograde amnesia. Patients with this disorder experience an impairment of memory retention that is greater for more recently acquired memories (Squire and Alvarez, 1995). For instance, a patient with extensive MTL damage could recall spatial and navigational characteristics of a neighbourhood he lived in as a youth, but could not remember these same characteristics for the neighbourhood he currently inhabited (Teng and Squire, 1999). Thus, memory traces usually consolidate over years in humans to become independent of the memory processing systems that originally initiated the consolidation process.

c) Hippocampal Memory System

*i. Information flow*

Declarative memory is the type of memory referred to in everyday language, and involves the conscious recollection of facts and events (Graf and Schacter, 1985; Squire and Zola, 1996). Studies of amnesia and surgical ablation show that this type of memory is dependent on structures in the MTL, particularly the hippocampus (Eichenbaum and

Cohen, 2001; Zola-Morgan et al., 1986). But how does information entering the brain become a long-lasting declarative memory?

Information from the external environment is first processed in primary cortical areas that are specialized for particular sensory modalities and motor functions. After multiple stages of processing, the information is passed to association areas in the prefrontal, parietal, and temporal lobes (Eichenbaum and Cohen, 2001). These association areas synthesize data from different sensory modalities and send dense projections to the MTL. Within the MTL, the parahippocampal region is a key locus of convergence for neocortical input (Eichenbaum, 2000). Information in the parahippocampal region is accumulated through a series of interconnections and subsequently projected from the entorhinal cortex to the hippocampus (Lavenex and Amaral, 2000; Mesulam, 1998).

After passing through the circuitry of the hippocampus, information is sent back to the parahippocampal region. Outputs of the parahippocampal region project to the neocortical areas that originally generated the inputs (Burwell, 2001; Eichenbaum and Cohen, 2001). These anatomical connections establish bidirectional data streams between the hippocampus and neocortical association areas. As such, the hippocampus is ideally situated to survey incoming data and selectively mediate long-term storage of salient information. The hippocampus is also reciprocally connected to several subcortical areas via an axon bundle known as the fornix. This pathway communicates information about behavioural state, including arousal, attention and emotion, via important neurmodulatory projections (Cassel et al., 1997; Eichenbaum and Cohen, 2001).

*ii. Hippocampal circuitry*

The hippocampus is separated into histologically distinct subregions thought to differentially contribute to information processing and memory function (**Figure 1.2**). These subregions are termed the dentate gyrus, area CA3, area CA1, and the subiculum. Whereas all subregions receive direct subcortical input, information from the cortex flows sequentially through the hippocampal circuitry (Johnston and Amaral, 2004). Projections from the parahippocampal region follow either 'long' or 'short' routes (Eichenbaum, 2002). The long route is also known as the trisynaptic circuit because it is comprised of three excitatory pathways within the hippocampus. The perforant pathway originates in the entorhinal cortex of the parahippocampal region and terminates on the granule cells of the dentate gyrus. The granule cells extend axons to area CA3 in the mossy fibre projection. Principal cells in area CA3 then project via the Schaeffer collaterals to principal cells in area CA1. The circuit is completed by connections from area CA1 to the subiculum, and projections from both these areas back to the parahippocampal region. The short connection route involves direct connections from the parahippocampal region to area CA1 and the subiculum.

Although many studies treat the hippocampus as a unified entity, evidence from neural network organization, lesions, and computational modeling suggest that hippocampal subregions display specificity of function. For instance, area CA3 is associated with spatial pattern completion and autoassociation, whereas area CA1 functions in temporal pattern completion and association (Kesner et al., 2004; Treves and

Rolls, 1994). Further investigation of subregion specialization is required to fully understand the intrinsic processing that occurs in the hippocampus.

*iii. Theories of hippocampal function*

The anatomy and circuitry of the hippocampus are conducive to information processing. Correspondingly, evidence from both animal and human studies suggests that the hippocampus is crucial for declarative memory (Eichenbaum and Cohen, 2001). For example, patient R.B. became amnesic after an ischemic episode, and could not form new memories of people, places, or events. Post-mortem histology demonstrated damage restricted to the hippocampus (Zola-Morgan et al., 1986). Although the details remain somewhat controversial, the hippocampus is thought to participate in spatial and temporal processing necessary for declarative memory.

Because certain cells in the hippocampus are activated in response to an animal's spatial position (place cells), the hippocampus has been hypothesized to create spatial maps necessary for spatial memory (O'Keefe and Nadel, 1978). In humans, recalling complex spatial routes or navigating through a virtual environment induces activation in the parahippocampal region and hippocampus (Hartley et al., 2003; Maguire et al., 1996). Studies using rats demonstrate that lesions of the hippocampus impair performance on spatial memory tasks. For instance, rats can normally be trained to remember the location of a submerged platform hidden in a pool of milky water in a task known as the Morris water maze (Morris et al., 1982). Hippocampus-lesioned animals fail to locate the platform when they are placed in the pool from different starting positions (Eichenbaum et al., 1990).

Similarly, memory for contextual fear conditioning requires the hippocampus (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Selden et al., 1991). Contextual fear conditioning is performed by placing a rodent in a novel environment and subsequently administering a fearful stimulus, such as a footshock (Maren, 2001). The animal then displays behaviours associated with fear (including freezing, increased heart and respiration rate, and enhanced auditory startle) when returned to the context, as a result of its memory for the footshock in that environment (Davis et al., 1993; Maren, 2001). Lesions of the hippocampus disrupt this spatial conditioning and result in an absence of fear when the animal is re-exposed to the context (Holland and Bouton, 1999; Phillips and LeDoux, 1992). The hippocampus may therefore facilitate the separation of spatial patterns, establishing memory for the precise localization of objects in space and associating events with specific spatial contexts.

Memory for the temporal order of events is also dependent on the hippocampus. The hippocampus does not mediate STM for odors in rats, but is involved in memory for the sequence of odors (Otto and Eichenbaum, 1992). The contribution of the hippocampus to the formation of associations between spatial and temporal representations has led some to hypothesize a general role for the hippocampus in relational memory. Eichenbaum and Cohen (2001) proposed that the hippocampus stores memories in a way that allows for the flexible and inferential use of these memories. As such, the hippocampus could be required for acquiring and associating both spatial and non-spatial information within a representational framework (McNamara et al., 2003).

The hippocampus also mediates consolidation of declarative memory. When direct projections from the entorhinal cortex to the hippocampus are ablated 24 hours

after training on a spatial water maze task, rats display intact STM, but deficient LTM. Ablation of this pathway 3 weeks after training does not generate similar LTM deficits (Remondes and Schuman, 2004). Similarly, damage to the hippocampal region 1 day, but not 21 days, after training for social transmission of food preference impairs LTM (Ross and Eichenbaum, 2006). These studies support the view that the hippocampus cooperates with the neocortex to establish a permanent neocortical memory store. Once cortical connections are sufficiently strengthened, the memory can be accessed independently of the hippocampus (McClelland et al., 1995; Squire and Alvarez, 1995). However, some evidence implicates the hippocampus in permanent memory storage. Animals trained in the Morris water maze task at different time points prior to lesioning of the hippocampus display memory deficits that do not correlate with the training-lesion interval (Sutherland et al., 2001). Thus, the hippocampus may also be required for storage/retrieval of some memories.

*iv. Hippocampal networks*

Hebb (Hebb, 1949) suggested that coordinated activity in assemblies of neurons could underlie encoding and processing of cognitive information. In the hippocampus, network-level coding units, also known as neural cliques, have been discovered (Lin et al., 2006). These neural cliques suggest a mechanism for memory encoding and recall. For example, ensemble recordings from large numbers of neurons in area CA1 reveal differential cell firing in response to various experiences (Lin et al., 2005). Activity of neurons within experience-specific neural cliques allows discrimination between these experiences, and thereby encodes the identity of the experience. Neural cliques may involve the activity-dependent modification of synaptic weights to form an attractor

network. An attractor network has preferred states, such that inputs to the system cause it to stably evolve into one of these states (Hopfield, 1982). In such a network, pattern completion (recall of memory from incomplete input stimuli) and pattern separation (segregation of different memories in the same network) are possible (Guzowski et al., 2004; Leutgeb et al., 2005). These network processes provide insight into real-time memory formation and identify potential systems level correlates for learning and memory.

### 3. Synaptic Plasticity

The discovery that neurons mediate signaling in the central nervous system led to the proposal that they may also possess the ability to store and retrieve information (Ramon y Cajal, 1893; Tanzi, 1893). In fact, Cajal suggested that connectivity between neurons was positively correlated with mental ability (1893). In 1949, Donald Hebb postulated that synaptic activity could elicit long-lasting changes in the excitability of neurons that comprise a sort of synaptic memory (Hebb, 1949). He stated:

"When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." pg. 62

In this manner, neurons were hypothesized to retain a record of their past activity and potentially mediate learning and memory.

#### a) Synaptic Plasticity and Behaviour in Aplysia

Although hypotheses about the neural mechanisms of memory existed in the 1800s, the complexity of the mammalian nervous system greatly hindered experimental investigation on a cellular level. Reductionist approaches provided the first major

advances in the understanding of cellular learning and memory. To this end, Eric Kandel and colleagues pioneered use of the marine invertebrate *Aplysia californica* (Kandel, 2001; Kandel et al., 1976). This model system provides key technical advantages compared to the more complicated mammalian systems: (1) fewer neurons, (2) larger neurons, (3) more easily identifiable and consistently located neurons.

Aplysia exhibit easily observable and modifiable behavioural responses. One of these responses, the gill-withdrawal reflex, allows the animal to protect its vulnerable gill and siphon from potentially noxious external stimuli. In the laboratory, application of a light tactile stimulus to the siphon or mantle of the animal elicits a measurable retraction of the gill. The strength of this behavioural response is determined by the animal's memory of previous stimuli, and thus is an indication of learning (Pinsker et al., 1973).

For instance, applying a noxious stimulus in the form of a tail shock induces sensitization of the Aplysia gill-withdrawal reflex. Subsequent stimulation of the siphon with a neutral stimulus elicits a gill withdrawal that is increased in both strength and duration (Dudai, 1989; Pinsker et al., 1973). Sensitization of this reflex can be either short-term or long-term, depending on the number of training sessions (tail shocks) given (Castellucci et al., 1989; Kandel, 2001; Pinsker et al., 1973).

Ordinarily, a stimulus to the siphon activates sensory neurons in the siphon skin. These sensory neurons excite motor neurons in the gill that can initiate the withdrawal movement. Synaptic input from sensory neurons to both excitatory and inhibitory interneurons also allows polysynaptic modulation of the resulting motor response (Dudai, 1989; Kandel, 2001). When a tail shock is applied, facilitating serotonergic interneurons are activated. These interneurons release serotonin (5-HT) onto the presynaptic terminals

of sensory neurons originating from the siphon. 5-HT increases transmitter release from the sensory neuron, generating a larger postsynaptic response in the motor neuron and consequently a stronger gill withdrawal (Glanzman et al., 1989; Mackey et al., 1989).

The simplicity and accessibility of this neural network permitted in-depth investigation of the cellular and molecular mechanisms involved in sensitization (Byrne et al., 1974; Castellucci et al., 1970; Hawkins et al., 1981). 5-HT receptors on the sensory neuron couple to guanine nucleotide-binding regulatory proteins (G-proteins) responsible for initiating signaling via the second messengers cAMP and phospholipase C. These second messengers activate cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), which act in concert to (1) mobilize neurotransmitter vesicles for release, (2) decrease potassium conductance to prolong action potentials, (3) enhance calcium ($Ca^{2+}$) conductance through voltage-gated calcium channels, and (4) facilitate $Ca^{2+}$-dependent transmitter release (Castellucci et al., 1980; Klein and Kandel, 1980). These cellular processes constitute presynaptic facilitation, and result in short-term sensitization (**Figure 1.3**).

Long-lasting sensitization of the gill-withdrawal reflex also relies on biophysical changes to the presynaptic sensory neuron. However, this process requires persistent protein kinase activity and macromolecular synthesis, ultimately resulting in structural modifications to the sensorimotor synapse (Bailey and Chen, 1983; Bailey and Chen, 1988; Castellucci et al., 1986; Greenberg et al., 1987; Schwartz et al., 1971; Sossin et al., 1994; Sweatt and Kandel, 1989). Repeated activation of 5-HT receptors recruits the mitogen-activated protein kinase (MAPK) cascade that acts conjointly with PKA to regulate the expression of genes under the transcriptional control of cAMP response

element binding protein (CREB) (Martin et al., 1997; Michael et al., 1998; Muller and Carew, 1998). The resulting proteins contribute to structural plasticity of the synapses and growth of new synaptic connections (Bailey et al., 2004; Bailey et al., 1992; Kim et al., 2003).

Cellular and behavioural studies of Aplysia demonstrate a direct relationship between synaptic plasticity and the acquisition and retention of implicit memory. Importantly, this reductionist approach established a framework for investigation of more complex systems. Numerous mechanisms of activity-dependent plasticity discovered in Aplysia are similarly observed in higher organisms.

b) Synaptic Plasticity in the Mammalian Brain

*i. Long-term potentiation*

Theoretical models and studies in Aplysia suggested that the experience-dependent alteration of neural connections could mediate information storage in the mammalian brain. However, experimental evidence of such a phenomenon was not obtained until the 1970s, when Bliss and Lomo discovered the long-term potentiation (LTP) of synaptic strength (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973). In response to high frequency electrical stimulation, synapses in the hippocampus of a rabbit displayed a persistent enhancement of synaptic transmission. This plastic change was thought to preserve a record of past neural activity, thereby encoding information.

Significant evidence supports the notion that LTP is responsible for memory formation and persistence in the brain (Bliss and Collingridge, 1993; Martin and Morris, 2002; Moser et al., 1998). Besides the noteworthy lack of an empirically well-supported rival theory, LTP also possesses numerous characteristics crucial for an information

storage system. Firstly, LTP can be extremely long-lasting. The initial documentation of LTP showed that it could persist for days (Bliss and Gardner-Medwin, 1973). LTP may last for up to a year (Abraham et al., 2002), and non-decremental LTP has been observed in area CA1 of the hippocampus (Staubli and Lynch, 1987) and in the neocortex (Trepel and Racine, 1998).

Importantly, LTP is pathway-specific. Synaptic alterations are limited to active synapses, and do not affect nearby inactive synapses (Andersen et al., 1977). This selectivity of response preserves the precision of information storage, and allows individual synapses to function as computational units (Bliss and Collingridge, 1993).

LTP also demonstrates the key properties of cooperativity and associativity. Activity in numerous presynaptic fibres is often required to sufficiently activate the postsynaptic fibre and induce LTP (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973; Malenka, 1991; McNaughton et al., 1978). The necessity of this presynaptic 'cooperation' ensures that only stimuli capable of surpassing a threshold of synaptic activation elicit LTP. However, the associativity of LTP allows weak stimuli at one input to induce LTP if temporally paired with strong stimulation to an independent input (Gustafsson et al., 1987; Levy and Steward, 1979; Stanton, 1996). In this manner, neurons can perform computations of inputs and generate LTP in response to cooperative, synchronous synaptic activity.

*ii. Long-term depression*

Synaptic connections can also be persistently weakened, a phenomenon termed long-term depression (LTD). LTD is thought to counterbalance enhancements of synaptic strength associated with LTP and prevent the saturation of synapses. As such, it

was proposed that if activity in a presynaptic cell consistently failed to activate the postsynaptic cell, the efficacy of the connection between these cells would decrease (Stent, 1973). Correspondingly, prolonged low frequency stimulation that is subthreshold for the induction of LTP does elicit LTD *in vitro* and *in vivo* (Dudek and Bear, 1992; Heynen et al., 1996; Milner et al., 2004). Specific forms of LTD are thought to be conducive to some types of information storage (Etkin et al., 2006; Manahan-Vaughan and Braunewell, 1999; Nakao et al., 2002).

*iii. Metaplasticity*

The probability of LTP or LTD induction is regulated by numerous extrinsic factors. External influences such as the level of synaptic inhibition, release of neuromodulatory substances, and prior synaptic activity can modulate the 'state' of a synapse, contributing to a phenomenon known as metaplasticity – the plasticity of plasticity (Abraham et al., 2001). This control of plasticity is hypothesized to serve two functions: homeostasis, and the integration of stimuli over an extended time course.

Homeostatic control of plasticity is necessary to sustain repeated episodes of information storage. By increasing the threshold for LTP induction at potentiated synapses, metaplastic processes can prevent saturation of synaptic strength. Similarly, synapses expressing LTD experience an increased threshold for subsequent LTD induction (Abraham and Bear, 1996). The existence of a sliding threshold that bi-directionally regulates the generation of plasticity was first proposed by Bienenstock, Cooper, and Munro, and has come to be known as the BCM theory (Bienenstock et al., 1982). Evidence for this model has been observed in the developing visual cortex. In light-deprived rats, activity in the visual cortex is low, decreasing the threshold for LTP

induction and concomitantly increasing the threshold for LTD (Kirkwood et al., 1996). The presence of light therefore influences the metaplastic state of the cortical synapses and alters the resultant modification of synaptic strength in response to a given stimulus.

Metaplasticity may also increase the temporal processing power of neurons. Whereas summation of synaptic potentials occurs over milliseconds, metaplastic integration of synaptic events could increase this time period by orders of magnitude (Abraham et al., 2001). As such, synapses could respond not only to immediate patterns of synaptic activity, but to slower changes in the external milieu. For instance, release of catecholamines can alter the biophysical properties of neurons (Gray and Johnston, 1987; Raman et al., 1996). Synaptic activity occurring in concert with catecholamine release could therefore elicit a different plasticity response compared to the same synaptic activity in the absence of catecholamines. Indeed, application of catecholamine receptor agonists shifts the frequency-response relationship to favour potentiation *in vitro* (Katsuki et al., 1997).

Metaplastic processes could increase both the potential for synaptic integration and the range of synaptic responses to stimulation. Although metaplasticity affects numerous aspects of synaptic plasticity, further investigation into this diverse array of phenomena is necessary to understand the ramifications for information storage in the mammalian brain.

c) Hippocampal Long-Term Potentiation

*i. LTP induction*

Stimulation Protocols

Numerous stimulation protocols have been developed *in vivo* and *in vitro* to induce LTP. Commonly, brief trains of high frequency electrical stimulation (HFS, for example, 100 Hz) are applied to elicit robust LTP. Although this tetanic stimulation is a reliable induction protocol, it does not resemble *in vivo* neuronal firing patterns. As such, efforts have been made to generate LTP with stimulation protocols that more closely mimic patterns of activity in the brain.

For instance, field potential oscillations in the range of 3-12 Hz are observed in the hippocampus. This so-called theta rhythm is produced by release of acetylcholine (ACh) and γ-aminobutyric acid (GABA) from rhythmically discharging cells in the medial septum and diagonal band (Petsche and Stumpf, 1962), as well as glutamate release from the entorhinal cortex (Alonso and Llinas, 1989). Application of electrical stimuli at theta frequency can induce LTP, depending on the duration of stimulation (Staubli and Lynch, 1987; Thomas et al., 1998). Endogenously, pyramidal neurons also fire high frequency bursts of two to seven spikes ("complex spikes") that are coordinated with the phases of theta rhythm (Buzsaki, 1986; Otto et al., 1991; Ranck, 1973). Application of short, high frequency bursts with an interburst interval of approximately 200 ms is thought to resemble natural complex spike activity (Buzsaki, 1986; Stewart et al., 1992). This theta-like stimulation facilitates the induction of LTP (Larson et al., 1986; Otto et al., 1991; Staubli and Lynch, 1987). These induction protocols, which are based on *in vivo* neural activity, are believed to be more physiological than tetanic stimulation.

Mechanisms

When a presynaptic hippocampal neuron is stimulated by electrical pulses, the excitatory neurotransmitter glutamate is released from the presynaptic terminal, diffuses across the synaptic cleft, and binds to postsynaptic glutamate receptors. One type of ionotropic glutamate receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR), contributes to postsynaptic depolarization by allowing influx of sodium ions when activated by glutamate. This depolarization removes the voltage-dependent magnesium ($Mg^{2+}$) blockade of another ionotropic glutamate receptor, the N-methyl-D-aspartate receptor (NMDAR) (Mayer et al., 1984; Nowak et al., 1984). Importantly, activation of NMDARs results in the influx of calcium ions into the postsynaptic cell (Gu et al., 1996). A critical increase in postsynaptic intracellular calcium levels via NMDARs is necessary for LTP induction (Collingridge et al., 1983; Lynch et al., 1983; Malenka et al., 1988).

The characteristics of AMPARs and NMDARs provide a cellular explanation for many properties of LTP. Weak, asynchronous presynaptic stimulation may release enough glutamate to activate AMPARs, but will not result in sufficient postsynaptic depolarization to activate NMDARs. Consequently, calcium cannot enter the postsynaptic cell, and LTP is not induced. However, if numerous presynaptic fibres are activated in close temporal and spatial proximity, the postsynaptic cell is more likely to become depolarized and permit activation of NMDARs. This synaptic cooperativity is a key property of LTP. Furthermore, postsynaptic depolarization elicited by a strong presynaptic stimulus may spread to nearby synapses, permitting NMDAR activation and consequent LTP induction at weakly active synapses. The resulting association between

the strong and weak synaptic inputs could represent a way of linking information within a single neuron. NMDARs therefore function as coincidence detectors, ensuring that LTP is induced reliably when there is simultaneous activation of both the pre- and postsynaptic neuron. Following initial receptor activation, LTP is usually described in terms of expression (early phase of LTP) and maintenance (late phase of LTP).

*ii. LTP expression*

A critical increase in calcium in the postsynaptic neuron initiates multiple intracellular signaling cascades that contribute to the expression of LTP. Although numerous signaling molecules are implicated in LTP, only certain molecules are both necessary and sufficient for LTP expression (Sanes and Lichtman, 1999; Soderling and Derkach, 2000). The mechanisms of this early phase LTP (E-LTP) involve enhancement of synaptic transmission by increasing conductance of receptors present in the synaptic membrane and insertion of additional receptors into the membrane (**Figure 1.4**).

$Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII), PKA and PKC have been demonstrated to phosphorylate specific residues of AMPA and/or NMDA receptors (Barria et al., 1997; Lee et al., 2000; Malenka and Bear, 2004; Roche et al., 1996). This phosphorylation alters the biophysical properties of the receptors, permitting increased channel conductance (Benke et al., 1998; Derkach et al., 1999). Pharmacological blockade of either CaMKII or PKC during LTP induction results in deficient potentiation that decays to baseline within minutes (Hu et al., 1987; Malinow et al., 1989). Transgenic mice engineered without endogenous CaMKII display comparable deficits in LTP expression (Silva et al., 1992).

Although PKA is activated by stimuli that induce LTP (Roberson and Sweatt, 1996), inhibiting PKA activity does not substantially disrupt E-LTP generated by one 100 Hz train (Abel et al., 1997; Blitzer et al., 1998; Duffy and Nguyen, 2003; Huang and Kandel, 1994). Phosphorylation downstream of PKA inactivates protein phosphatase 1 (PP1) (Hemmings et al., 1984) and subsequent suppression of PP1 activity promotes synaptic potentiation (Blitzer et al., 1998). In this manner, PKA can gate LTP expression by regulating activity of phosphatases (Blitzer et al., 1995; Blitzer et al., 1998; Woo et al., 2002).

Activation of these kinase cascades also facilitates addition of new receptors into the synaptic membrane. Expression of LTP is proposed to drive AMPAR trafficking to the membrane, thereby allowing synapses previously devoid of AMPARs ("silent" synapses) to participate in synaptic transmission (Kullmann, 2003; Poncer, 2003). Whereas enhanced CaMKII activity is sufficient to drive additional AMPARs into the membrane (Hayashi et al., 2000), PKA is necessary but not sufficient for this process (Esteban et al., 2003).

Importantly, the initial expression of LTP relies on covalent modification of previously existing proteins and does not require new macromolecular synthesis (Huang and Kandel, 1994; Reymann et al., 1985). These biochemical characteristics parallel the mechanisms of short-term facilitation in Aplysia (Montarolo et al., 1986). Both E-LTP and short-term facilitation are decremental, lasting only hours (Huang et al., 1996; Schwartz et al., 1971). The maintenance of plasticity requires additional synaptic modifications and this constraint is conserved between experimental models.

*iii. LTP maintenance*

LTP can persist for many hours *in vitro* (Frey et al., 1996; Huang et al., 1996) and months *in vivo* (Abraham et al., 2002). Late phase LTP (L-LTP) is often generated by applying multiple trains of HFS ( > 2 trains) and requires intracellular signaling that elicits new mRNA and protein synthesis (Deadwyler et al., 1987; Nguyen et al., 1994; Stanton and Sarvey, 1984). Interestingly, studies investigating the effects of transcription and translation inhibitors on L-LTP suggest that the requirement for transcription and/or translation is not constant over time. Whereas inhibition of translation generates an early, progressive decay of potentiation, blockade of transcription produces a delayed decay, usually beginning approximately an hour after LTP induction (Frey and Morris, 1997; Frey et al., 1988; Frey et al., 1996; Nguyen et al., 1994). These kinetic patterns suggest that L-LTP is initially dependent on translation, but independent of transcription. Transcription becomes necessary to sustain L-LTP at a later time point (Kelleher et al., 2004b).

Transcriptional Regulation of L-LTP

Application of transcription inhibitors prevents the long-term maintenance of LTP (Frey et al., 1996; Nguyen et al., 1994). Similarly, blocking the transfer of mRNA and proteins from the cell body to the dendrites by severing the neurons results in decay of LTP (Frey et al., 1989). Thus, transcription is required for the consolidation of some forms of long-lasting LTP.

Activity-dependent initiation of transcription is mediated by kinase cascades. For instance, interaction of PKA and/or PKC with MAPK leads to the phosphorylation of a transcription factor known as CREB (Impey et al., 1996; Impey et al., 1998; Roberson et

al., 1999). Blockade of PKA or MAPK inhibits both L-LTP and CREB phosphorylation (Davis et al., 2000; Matsushita et al., 2001; Shaywitz and Greenberg, 1999; Sweatt, 2004). CREB subsequently binds cAMP-responsive elements on immediate early genes (IEGs) and stimulates their transcription (Lee and Masson, 1993). Stimulation patterns that induce L-LTP generate increases in IEG expression, whereas subthreshold stimulation does not induce similar levels of IEG expression (Dragunow et al., 1989; Richardson et al., 1992).

The involvement of the nucleus in L-LTP poses an important question: how is the synaptic input specificity of LTP preserved when mRNA required for LTP maintenance is generated centrally at the cell body and potentially available to all synapses? One proposal suggests that active synapses are somehow 'tagged', allowing only these synapses to capture and utilize gene products distributed from the nucleus (Frey and Morris, 1997; Frey and Morris, 1998; Sossin, 1996). Recently, the discovery that mRNAs can be locally translated within dendrites has shed new light on the transcriptional and translational regulation of L-LTP (**Figure 1.5**).

Translational Regulation of L-LTP

The first evidence of dendritic translation came with the discovery that polyribosome complexes are localized in dendrites (Steward and Schuman, 2001; Steward and Schuman, 2003). Recent studies suggest that components of translational machinery are constitutively present in dendrites, and can be mobilized in response to synaptic activity (Ostroff et al., 2002; Smart et al., 2003; Tang and Schuman, 2002). As such, dendritic translation could affect maintenance of synaptic plasticity by synthesizing

protein products specifically at active synapses, and/or creating a synaptic tag to permit capture of gene products synthesized elsewhere (Sutton and Schuman, 2005).

Furthermore, signaling cascades required for long-term synaptic plasticity regulate translational processes (**Figure 1.6**). Activation of the extracellular-signal regulated kinase (ERK) and mammalian target of rapamycin (mTOR) cascades are necessary for L-LTP (Cammalleri et al., 2003; English and Sweatt, 1997; Tang et al., 2002). These kinase cascades also control protein synthesis via phosphorylation of kinases and translation factors involved in translation initiation (Banko et al., 2006; Kelleher et al., 2004b).

Interestingly, activation of various neuromodulatory receptors in the hippocampus leads to local protein synthesis. Application of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 elicits L-LTP that requires dendritic protein synthesis (Kang and Schuman, 1996). Similarly, stimulation of metabotropic glutamate receptors (mGluRs) induces late-phase LTD (L-LTD) that is insensitive to transcriptional inhibition, but dependent on translation (Huber et al., 2001). Activated neuromodulatory receptors may therefore contribute to some forms of synaptic plasticity by influencing the machinery used for dendritic translation of mRNA.

mRNAs coding for cytoskeletal proteins, signaling kinases and receptor subunits are localized in dendrites (Steward and Schuman, 2001). Some of these mRNAs, such as the fragile X mental retardation protein (FMRP) are synthesized locally in response to synaptic stimulation (Weiler et al., 1997). The importance of local protein synthesis in information processing has recently been addressed in Drosophila melanogaster. Translation of CaMKII mRNA occurs at postsynaptic sites during induction of LTM,

producing patterns of local protein synthesis that are specific to the memory (Ashraf et al., 2006). As such, local protein synthesis is strongly linked to synaptic plasticity and implicated in memory, although much remains to be clarified concerning the regulation, selectivity, and specificity of this process.

**4. Synaptic Plasticity and Memory**

Activity-dependent alteration of synaptic strength is hypothesized to underlie certain forms of learning and memory in the mammalian brain (Bliss and Collingridge, 1993). Various experimental approaches are employed with the goal of ultimately establishing a causal link between synaptic plasticity and memory. Although substantial correlative evidence currently exists, the definitive role of long-lasting modifications of synaptic strength in memory formation and retention remains elusive (Martin and Morris, 2002; Morris, 2003). Several lines of investigation are currently being pursued to shed light onto this issue.

a) Pharmacologic and Genetic Studies

Pharmacologic manipulations demonstrate that many of the molecules and processes required for memory are also essential for synaptic plasticity. This correlative approach suggests that phases of memory mechanistically resemble phases of plasticity. STM generally shares molecular mechanisms with E-LTP, whereas LTM shares mechanisms with L-LTP. For instance, both LTM and L-LTP require new macromolecular synthesis (Agranoff, 1967; Davis and Squire, 1984; Flexner, 1966; Izquierdo and McGaugh, 2000; Stanton and Sarvey, 1984). Transcription and translation

are needed for the consolidation of memory and the maintenance of LTP, indicating an important connection between long-term information storage on cellular and behavioural levels. Furthermore, key signaling processes, such as CREB phosphorylation, enhance both L-LTP and learning (Barco et al., 2002; Genoux et al., 2002).

Genetic experimentation also implies a correlative link between synaptic plasticity and memory. One of the most convincing correlations between learning and LTP is demonstrated in transgenic mice that lack NMDA receptors in area CA1 of the hippocampus (Tsien et al., 1996). These mice do not display NMDA receptor currents, and consequently do not exhibit LTP in area CA1. Pyramidal neurons in this subregion also develop impaired spatial representations, as evidenced by decreased spatial specificity of place fields (McHugh et al., 1996). Behaviourally, these mice have deficient hippocampus-dependent spatial memory as tested by using the Morris water maze, but perform normally on non-spatial memory tasks and show no sensorimotor or motivational impairments (Tsien et al., 1996). Therefore, these studies demonstrate that the targeted deletion of receptors crucial for LTP induction results in inhibition of appropriate place cell activity and spatial memory, while leaving other cognitive processes intact.

Transgenic mice have also been developed that exhibit selective deficits in hippocampal-dependent LTM and hippocampal L-LTP. Inhibition of PKA by expression of a dominant-negative PKA subunit in the postnatal forebrain results in mice does not affect E-LTP and STM. However, this genetic alteration prevents maintenance of L-LTP induced by multiple trains of HFS, and correspondingly produces impaired LTM for contextual fear conditioning and spatial navigation (Abel et al., 1997). These findings

strongly support the notion that synaptic plasticity underlies memory in the mammalian brain.

b) Occlusion Studies

If LTP is required for memory, then LTP induction at massive numbers of synapses should occlude subsequent learning. One of the first studies to test this hypothesis was conducted by applying brief, high frequency stimulation to the perforant pathway of the hippocampus in chronically prepared animals (McNaughton et al., 1986). This stimulation protocol resulted in a persistent increase of synaptic strength measured extracellularly. Animals that underwent this procedure could not acquire new spatial information, although they performed normally on tasks requiring working memory or previously well-established memory. Furthermore, animals with no residual capacity for LTP display disrupted spatial memory, whereas animals with as little as 10% are unimpaired (Moser et al., 1998).

Exogenous LTP induction after training on a memory task has also been shown to impair spatial memory. Application of HFS to the hippocampi of animals trained in spatial navigation of the water maze impaired memory retention in a probe trial administered at a later time point (Brun et al., 2001). Importantly, application of an NMDA receptor antagonist during HFS prevented memory deficits. Therefore, HFS has the potential to disrupt memory only when it can alter synaptic strength.

c) Detection Studies

Detection of changes in synaptic strength following learning more clearly demonstrates the role of synaptic plasticity in memory. Learning-induced alterations can

be difficult to observe, potentially because information may be stored over spatially distributed synapses and involve both increases and decreases in synaptic strength (Hosokawa et al., 2003). However, such alterations have been detected in the amygdala, motor cortex, and hippocampus, lending support to the synaptic plasticity theory of memory.

*i. Amygdala*

The amygdala is necessary for a memory task known as cued fear conditioning (Maren, 2001; Romanski and LeDoux, 1992). In this paradigm, an animal receives a neutral conditioned stimulus (CS) such as a tone, paired with an aversive unconditioned stimulus (US) such as an electric shock. Subsequent presentation of the CS elicits defensive and fearful behavioural responses in the animal. Because the neural circuitry underlying this form of learning is well characterized, substantial progress has been made in determining its cellular mechanisms.

Rogan and colleagues (Rogan et al., 1997) first demonstrated induction of LTP following learning in the amygdala. Presentation of an auditory CS evokes field potentials in the lateral amygdala (LA), allowing measurements of synaptic strength after various training protocols. They found that paired CS/US training in rats generates memory and concomitant fear of the CS, whereas unpaired CS/US training does not. Accordingly, LTP is induced in the LA only after paired CS/US training. Similarly, *in vitro* excitatory postsynaptic potentials evoked in the LA are larger in fear-conditioned rats compared to untrained rats (McKernan and Shinnick-Gallagher, 1997). Thus, increased efficacy of LA synapses responsible for CS processing is involved in memory for fear conditioning in rats.

*ii. Motor cortex*

Motor cortical representations have the capacity for substantial experience-dependent reorganization (Buonomano and Merzenich, 1998; Sanes and Donoghue, 2000). For instance, training rats in a skilled reaching task induces expansion of areas in the motor cortex consistently activated by the task (Kleim et al., 1998). Rioult-Pedotti and colleagues (Rioult-Pedotti et al., 1998) subsequently demonstrated that this motor skill learning results in strengthened synaptic connections in the trained motor cortex relative to untrained motor cortex. Furthermore, this learning-induced enhancement of synaptic transmission partially occluded artificial induction of LTP. These findings suggest that LTP-like effects are responsible for learning in the motor cortex.

*iii. Hippocampus*

Despite numerous studies examining hippocampus-dependent memory and hippocampal synaptic plasticity, it remains a challenge to observe alterations of synaptic strength in response to learning. A recent study provides evidence that such alterations can occur in the hippocampus after associative conditioning. Gruart and colleagues (Gruart et al., 2006) trained mice on a hippocampus-dependent associative memory task and concurrently measured evoked field potentials in area CA1 of the hippocampus. They found that the strength of synaptic connections increased with conditioning, and decreased during memory extinction. Such modifications were not observed in mice that did not undergo conditioning. Furthermore, application of an NMDA receptor antagonist prevented both memory for the task and synaptic changes observed during conditioning.

Overall, synaptic plasticity has been detected in response to memory formation and retention in various brain regions. Taken together with correlative evidence generated in pharmacologic, genetic, and occlusion studies, these results convincingly demonstrate synaptic plasticity is a likely cellular mechanism for some forms of memory in the mammalian brain.

## 5. Cellular Noradrenergic Neuromodulation

### a) Neuromodulation

Direct communication between neurons is accomplished by chemical synaptic transmission across synapses. Neuroactive agents involved in synaptic transmission are thought to mediate brief, spatially restricted, postsynaptic responses. However, some of these agents can also pre- or postsynaptically modulate neuronal responses without directly evoking postsynaptic potentials. This type of synaptic action is loosely classified as "neuromodulation." In general, neurotransmitters act through ligand-gated ion channels, whereas neuromodulators act through intracellular second messenger cascades to give slower, longer-lasting, and more spatially diffuse responses (Hasselmo, 1995). This distinction is becoming blurred with the discovery that specific neurochemicals can elicit effects either as fast neurotransmitters or through slower neuromodulatory mechanisms. Regardless of terminology, neuromodulators are responsible for numerous cellular responses that contribute to normal and pathological brain physiology.

### b) Noradrenaline Anabolism and Catabolism

Noradrenaline (NA) was first recognized as a potential neurotransmitter and neuromodulator in the central nervous system in 1954 (Vogt, 1954). Subsequently, it was found to modulate various cellular responses in neurons and has been implicated in multiple functions at the behavioural and neural systems levels.

NA, like dopamine (DA) and adrenaline, is a catecholamine comprised of an ethylamine group attached to a catechol ring (**Figure 1.7**). A common biosynthetic pathway, beginning with the amino acid precursor tyrosine, is used to manufacture these neurotransmitters (Nestler et al., 2001). The rate-limiting step in central NA synthesis is the enzyme tyrosine hydroxylase. Importantly, tyrosine hydroxylase activity is regulated by PKC, PKA and CaMKII, allowing for short-term alterations in rates of NA synthesis (Goldstein, 1995). Release occurs presynaptically by calcium-dependent exocytosis, and it is regulated primarily by presynaptic autoreceptors that can monitor concentrations of catecholamines at the synapse (Dixon et al., 1979). Once NA is released at synapses, local inactivation of NA is accomplished by efficient membrane transport-mediated reuptake. Monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) are the primary enzymes involved in degradation of NA into 3-methoxy-4-hydroxy-phenylethyleneglycol (MHPG) to terminate transmitter action (Cooper et al., 2003).

c) Noradrenergic Pathways in the Mammalian Brain

Noradrenergic cell bodies are located primarily in the locus coeruleus (LC) and lateral ventral tegmental fields (Cooper et al., 2003). The LC is a compact nucleus found bilaterally in the caudal pontine gray. It projects widely throughout the cerebral cortex, midbrain, cerebellum, and spinal cord (Moore and Bloom, 1979), with dense innervation in the thalamus, amygdala and hippocampus (Morrison and Foote, 1986). Furthermore,

axons from the LC branch out as they reach their target regions to widely innervate numerous cortical and subcortical structures (Holets, 1990).

The lateral ventral tegmental fields contain more diffusely scattered noradrenergic neurons. Axons from these neurons intermingle with those from the LC and may specifically innervate the basal forebrain regions, including the amygdala and septum (Barone et al., 1981; Cooper et al., 2003). The diverse projections of the noradrenergic neuromodulatory system support a role for NA in global information processing mediated by coordinated cellular effects in several brain regions (Berridge and Waterhouse, 2003).

d) Noradrenergic Receptor Subtypes

Specific membrane receptors on effector cells bind NA and consequently trigger physiological responses (**Figure 1.8**). NA receptors belong to a large family of receptors that couple to G-proteins to initiate intracellular signaling. However, activation of NA receptors can produce very different intracellular effects depending on the characteristics of the stimulated receptor. Based on these effects, receptors are broadly classified as α1–, α2-adrenergic and β-adrenergic. How activation of these divergent receptor types is coordinated to generate a physiological or behavioural outcome remains highly speculative.

*ι. α1-adrenergic receptors*

α1-Adrenergic receptors are distributed throughout the central nervous system (CNS). Subclassification of this adrenoceptor based on physiological actions and affinity for various pharmacological agents yields $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$ receptor subtypes (Harrison et al., 1991). These receptor subtypes are expressed to varying degrees in the hippocampus (Jones et al., 1985; Pieribone et al., 1994). Specifically, mRNA for the $\alpha_{1A/D}$ receptors is

present in the pyramidal neurons of the CA1–CA4 fields, and the hilar and granular neurons of the dentate gyrus (Day et al., 1997; Pieribone et al., 1994). Human studies have revealed a more precise distribution of α1-adrenergic subtypes. Receptors are restricted to area CA3 and the dentate gyrus, with $\alpha_{1A}$-adrenergic receptors concentrated in area CA3 and $\alpha_{1B}$-adrenergic receptors localized mostly in the molecular layer of the dentate gyrus (Zilles et al., 1991). α1-Adrenergic receptors are also found in glia (Lerea and McCarthy, 1989), and in populations of interneurons, where they appear to colocalize with somatosatin (Szot et al., 2005).

In general, α1-adrenergic receptors couple to the Gq/G11 form of G-protein. Activating this G-protein initiates signaling through phospholipase C, resulting in the production of the second messengers diacyglycerol (DAG) and inositol triphosphate (IP3). These second messengers mediate diverse effects on cellular metabolism. Importantly, both DAG and IP3 can increase intracellular calcium levels (Cotecchia et al., 1990; Sirvio and MacDonald, 1999).

Because α1-adrenergic receptors are expressed in multiple cell types in the hippocampus, activation of α1-adrenergic receptors can generate a variety of cellular effects depending on the target cell population.

Excitability of Principal Neurons

Activation of α1-adrenergic receptors decreases the excitability of principal neurons in the dentate gyrus (Harley, 1991), area CA3, (Pang and Rose, 1987) and area CA1 (Mynlieff and Dunwiddie, 1988) of the hippocampus. This lowered propensity to fire action potentials is often evident as decreased amplitude of the extracellular population spike. The α1-adrenergic receptor-mediated hyperpolarization of principal neurons may result at least partially from activation of inhibitory interneurons. However, there is some

evidence that α1-adrenergic receptors can transiently potentiate the amplitude of the population spike in the dentate gyrus *in vivo* (Chaulk and Harley, 1998).

Interestingly, endogenous release of NA from the LC initially suppresses activity of pyramidal neurons before activating them. α1-Adrenergic receptors are responsible for the period of suppression, whereas β-adrenergic receptors are responsible for the period of activation (Curet and de Montigny, 1988b). This biphasic sequence of inhibition-excitation may be related to *in vitro* observations that high concentrations of applied NA decrease pyramidal cell excitability, presumably by preferential activation of α1-adrenergic receptors. Correspondingly, lower concentrations of NA increase excitability, resembling β-adrenergic receptor activation (Mueller et al., 1981; Mynlieff and Dunwiddie, 1988; Rutecki, 1995). It is possible that varying concentrations of NA in the synaptic cleft allow α1- and β-adrenergic effects to predominate during different patterns of release.

Synaptic Plasticity of Principal Neurons

The effects of α1-adrenergic receptors on synaptic plasticity in the hippocampus are generally weak and indirect. These responses vary depending on the hippocampal subregion. In the dentate gyrus, α1-adrenergic agonists do not produce consistent changes in the slope of the extracellular postsynaptic potential (EPSP) *in vivo* (Chaulk and Harley, 1998).

In area CA3, presynaptic α1-adrenergic receptors are involved in presynaptic inhibition. Application of NA decreases release of glutamate (Glu) from presynaptic terminals, and this effect is blocked by an α1-adrenergic receptor antagonist (Scanziani et al., 1993). Because LTP at mossy fibre synapses has a presynaptic locus (Reid et al., 2004; Zalutsky and Nicoll, 1990), these receptors could affect LTP induction in this subregion.

Activation of α1-adrenergic receptors mediates diverse responses in area CA1. α1-Adrenergic agonists subtly facilitate LTP induction and maintenance when paired with weak

electrical tetanus (Izumi and Zorumski, 1999; Pussinen and Sirvio, 1998). The effects of NA on stability of LTP in area CA1 also have an α1-adrenergic component. NA application prevents the reversal, or depotentiation, of LTP by low-frequency stimulation (LFS). Activation of both α1- and β-adrenergic receptors is required to generate this immunity to depotentiation (Katsuki et al., 1997).

These weak α1-adrenergic effects could be explained by evidence suggesting that α1-adrenergic activation can either antagonize or potentiate β-adrenergic effects based on interactions with subtypes of adenylyl cyclase. α1-Adrenergic receptor activation alone produces no change, or a decrease in levels of cAMP. However, co-activation of α1- and β-adrenergic receptors increases activity of adenylyl cyclase (Daly et al., 1980; Pedarzani and Storm, 1996; Perkins and Moore, 1973). The subtle effects of α1-adrenergic receptors on LTP in CA1 could result from concurrent β-adrenergic receptor activation.

α1-Adrenergic receptors are also involved in LTD in area CA1. Application of high concentrations of NA or α1-adrenergic agonists induces a persistent decrease in synaptic strength (Scheiderer et al., 2004). The mechanism responsible for this synaptic response is unknown, but could include IP3-mediated calcium transients.

Glia

Glial cells can influence synaptic transmission and contribute to some forms of synaptic plasticity (Mazzanti et al., 2001). Activation of α1-adrenergic receptors induces calcium transients in hippocampal astrocytes (Duffy and MacVicar, 1995), suggesting that α1-adrenergic receptors regulate synaptic function by affecting these non-neuronal cells. Possible outcomes of astrocytic calcium flux include buffering of potassium, neurotransmitter uptake or release, and alterations in gene expression (Duffy and MacVicar, 1995).

Interneurons

Application of NA to area CA1 causes an α1-adrenergic receptor-dependent depolarization of interneurons in all cell layers (Bergles et al., 1996; Parra et al., 1998). Because many of these interneurons make synaptic connections throughout stratum pyramidale, they could potentially influence firing rates of pyramidal neurons (Bergles et al., 1996). Furthermore, activation of α1-adrenergic receptors on glutamatergic afferents to interneurons depresses feed-forward inhibition (Doze et al., 1991). Functionally, interneurons often respond to several neuromodulators, indicating that interactions between neuromodulatory systems can regulate these inhibitory networks (Parra et al., 1998).

*ii. α2-adrenergic receptors*

Based on sensitivity to pharmacological agents and tissue distribution, α2-adrenergic receptors can be further divided into $\alpha_{2A}$, $\alpha_{2B}$, and $\alpha_{2C}$ receptor subtypes (Harrison et al., 1991). The hippocampus contains roughly similar $\alpha_{2A}$ and $\alpha_{2C}$ receptor expression, whereas $\alpha_{2B}$ receptors are restricted to the diencephalon (Happe et al., 2004; Nicholas et al., 1996; Zeng and Lynch, 1991). The distribution of α2-adrenergic receptors is similar throughout all hippocampal subregions, with receptors localized predominantly presynaptically. However, some receptor expression is observed in dendritic spines of pyramidal and granule cells, as well as in glial processes (Milner et al., 1998). Therefore, several possible sites for α2-adrenergic receptor-mediated effects exist.

The α2-adrenergic receptor couples to the inhibitory G-protein Gi to reduce adenylyl cyclase activity and subsequently decrease intracellular cAMP (Dismukes and Mulder, 1976; Ruffolo et al., 1994; Summers and McMartin, 1993). In general, this signaling inhibits the electrical activity of pyramidal neurons. α2-Adrenergic agonists applied microiontophoretically to hippocampal areas CA1 and CA3 strongly suppress firing

of pyramidal neurons (Curet and de Montigny, 1988a). Studies examining endogenous NA release elicited by stimulation of the LC suggest that postsynaptic α2-adrenergic receptors exert this suppressant effect extrasynaptically (Curet and de Montigny, 1988b).

However, α2-adrenergic effects on hippocampal plasticity are primarily presynaptically mediated. Presynaptic α2-adrenergic autoreceptors are the main regulators of NA release, preventing further release when bound by NA diffusing from the synaptic cleft (Dixon et al., 1979). Additional signal transduction mechanisms may be recruited by α2-adrenergic receptors to fulfill this function, because α2-adrenergic receptor-mediated inhibition of transmitter release is insensitive to the inactivation of Gi proteins (Cooper et al., 2003).

α2-Adrenergic receptors are also involved in presynaptic inhibition of other neurotransmitters. Application of α2-adrenergic receptor agonists decreases excitatory Glu currents by reducing presynaptic Glu release (Boehm, 1999). This effect is blocked by inactivation of Gi/o proteins, and is mediated by a reduction in $Ca^{2+}$ currents through voltage-dependent calcium channels (VDCCs) (Boehm, 1999).

Overall, α2-adrenergic receptors appear to be critical for homeostatic and neuroprotective functions in the hippocampus, rather than mediating direct effects on synaptic plasticity. α2-Adrenergic receptors are implicated in inhibition of the spread of epileptic activity (Gellman et al., 1987) and protection during cerebral ischemia (Hoffman et al., 1991). Coupled with their suppressant effect on hippocampal pyramidal cells, these receptors could prevent neurotoxicity associated with excessive cell firing and release of excitatory neurotransmitters.

*iii. β-adrenergic receptors*

β-Adrenergic receptors are classified into β1-, β2- and β3-adrenergic subtypes based on differential potency of agonists. The agonist isoproterenol activates β1- and β2-adrenergic receptors more strongly than endogenous NA or adrenaline whereas β3-adrenergic receptors respond most strongly to NA. Furthermore, adrenaline is more potent than NA at β2-adrenergic receptors only (Cooper et al., 2003).

Expression of β-adrenergic receptors in the CNS is localized to the cerebral cortex, hippocampus, thalamus and cerebellum (Nicholas et al., 1993; Wanaka et al., 1989). In the human brain, the concentration of β-adrenergic receptors is highest in the subregions of the hippocampus (Reznikoff et al., 1986). β1- and β2-adrenergic receptors are localized in pyramidal cells and dentate granule cells, with β2-adrenergic receptor mRNA more prevalent than β1-adrenergic receptor mRNA. Whereas interneurons generally do not express β-adrenergic receptors (Hillman et al., 2005; Nicholas et al., 1993), isolated astrocytes in CA1 predominantly contain β2-adrenergic receptors (Zhu and Kimelberg, 2004). Evidence of functional β3-adrenergic receptors in this brain region is lacking (Hillman et al., 2005; Pupo and Minneman, 2001). β-Adrenergic receptors may therefore potently and directly affect plasticity in the hippocampus based on their primary association with the principal neurons in hippocampal subregions.

β-Adrenergic receptors couple to the Gs form of G-protein to mediate intracellular signaling (Minocherhomjee and Roufogalis, 1982; Raymond, 1995). Gs proteins stimulate adenylyl cyclase activity to increase levels of cAMP (Duman and Nestler, 1995). Correspondingly, application of selective β-adrenergic agonists induces an accumulation of cAMP in multiple brain regions. This β-adrenergic response involves primarily β1-adrenergic receptors in the cerebral cortex and hippocampus; a more significant β2-adrenergic contribution is observed in the cerebellum (Daly et al., 1981). Through

increases in intracellular cAMP, β-adrenergic receptors importantly modulate numerous processes involved in plasticity.

Channel Function

Functional modulation of gated membrane channels underlies some forms of hippocampal synaptic plasticity (Benke et al., 1998; Lee et al., 2003). Stimulating β-adrenergic receptors during excitatory synaptic transmission can increase the influx of calcium into the postsynaptic cell through NMDA receptors (Raman et al., 1996). This influx of calcium is critical for the initiation of signaling through intracellular kinase cascades such as PKC and CaMKII (Malenka and Bear, 2004). β-Adrenergic receptors further regulate calcium dynamics in dendrites by altering the properties of VDCCs (Cloues et al., 1997; Fisher and Johnston, 1990; Gray and Johnston, 1987). Application of β-adrenergic agonists enhances the activity of VDCCs in the dentate gyrus, area CA3, and area CA1 (Fisher and Johnston, 1990; Gray and Johnston, 1987; Hoogland and Saggau, 2004). Some evidence suggests that β-adrenergic receptor activation may also prevent the delayed facilitation of L-type VDCCs, contributing to the β-adrenergic receptor-mediated inhibition of the slow after-hyperpolarization current (Cloues et al., 1997). Thus, β-adrenergic receptors play a key role in the temporal dynamics of calcium flux in dendrites of hippocampal neurons.

Furthermore, β1-adrenergic-activated signaling modulates AMPA receptor function by phosphorylation of the GluR1 subunit (Vanhoose and Winder, 2003; Vanhoose et al., 2006). This subunit is critically involved in LTP (Shi et al., 2001; Zamanillo et al., 1999), and its regulation by β1-adrenergic receptors highlights the potential importance of these receptors to homosynaptic plasticity (Bailey et al., 2000). β-Adrenergic receptors also decrease spike frequency adaptation by blocking calcium-activated potassium channels that contribute to the afterhyperpolarization current (Haas and Rose, 1987; Madison and Nicoll,

1986; Pedarzani and Storm, 1996). The resulting increase in excitability of hippocampal neurons may underlie the ability of NA to enhance attention and arousal (McCormick et al., 1991).

Receptor Desensitization

β-Adrenergic receptors can also desensitize. PKA and β-adrenergic receptor kinase can phosphorylate β-adrenergic receptors and uncouple them from their associated Gs-protein. The receptor is then bound by β-arrestin, which competes with the Gs-protein and blocks activation of adenylyl cyclase. Internalization and sequestration of these uncoupled β-adrenergic receptors reduces the cellular response to NA or applied agonists (Cooper et al., 2003). Desensitization is particularly important in the noradrenergic neuromodulatory system because chronic administration of some psychiatric drugs affects endogenous levels of NA and its receptors (Garcia et al., 2004; Szabo and Blier, 2001).

Excitability

Activation of β-adrenergic receptors generally increases the excitability of principal neurons in all subregions of the hippocampus. In the dentate gyrus, application of NA or a β-adrenergic agonist causes long-lasting potentiation of the population spike, and NA depletion decreases the amplitude of the population spike (Neuman and Harley, 1983; Stanton and Sarvey, 1985; Stanton and Sarvey, 1987). The effects of β-adrenergic receptor activation in this subregion are also pathway specific. β-Adrenergic receptor-mediated potentiation is observed in the medial perforant path, whereas β-adrenergic receptor-mediated depression is seen in the lateral perforant path (Dahl and Sarvey, 1989). These pathways are histochemically and anatomically distinct, suggesting that differential effects of NA in this subregion may be important for selective information processing (Dahl and Sarvey, 1989; Lanthorn and Cotman, 1981).

Endogenous NA release from the LC similarly enhances cellular excitability in the dentate gyrus (Harley and Milway, 1986; Harley and Sara, 1992), and this response is mediated by both α– and β-adrenergic receptors (Chaulk and Harley, 1998). Interestingly, blockade of β-adrenergic receptors during high frequency electrical stimulation prevents LTP of the EPSP slope, but not potentiation of the population spike (Munro et al., 2001). This indicates that β-adrenergic receptors can differentially affect specific components of plasticity.

β1-Adrenergic receptors also increase potentiation of the pyramidal cell population spike in areas CA3 and CA1 (Dunwiddie et al., 1992; Heginbotham and Dunwiddie, 1991; Jurgens et al., 2005; Mueller et al., 1981). This enhanced cellular excitability increases the frequency of spontaneous bursts in area CA3 (Jurgens et al., 2005), potentially facilitating the auto-associative properties of this hippocampal subregion (Hasselmo, 1995).

*iv. β-adrenergic receptors and synaptic plasticity*

Long-term potentiation (LTP) and long-term depression (LTD) of synaptic strength are importantly linked to memory function in the mammalian brain (Braunewell and Manahan-Vaughan, 2001; Martin and Morris, 2002). β-Adrenergic receptors strongly modulate these forms of plasticity in all hippocampal subregions. However, the specific cellular responses elicited often depend on the histology, cellular circuitry, and biochemical properties of the subregion in question.

Dentate Gyrus

LTP generated by HFS requires β-adrenergic receptor activation in the dentate gyrus (Bramham et al., 1997; Munro et al., 2001). Application of a β-adrenergic antagonist prevents induction of LTP in the medial and lateral perforant paths (Bramham et al., 1997). However, β-adrenergic receptor blockade inhibits only HFS-induced potentiation of the EPSP, without affecting potentiation of the population spike (Munro et al., 2001). This

dichotomy suggests that distinct mechanisms underlie potentiation of synaptic strength and cellular excitability.

Paralleling β-adrenergic receptor-mediated alterations in population spike amplitude, application of NA or β-adrenergic agonists induces long-lasting potentiation of EPSPs in the medial perforant path, and long-lasting depression of EPSPs in the lateral perforant path (Dahl and Sarvey, 1989; Dahl and Sarvey, 1990). This plasticity requires activation of NMDA receptors, but not electrical activation of afferent neurons (Dahl and Sarvey, 1990).

Initial *in vivo* studies failed to find alterations in synaptic strength in response to NA or LC activation (Harley et al., 1989; Harley and Milway, 1986; Neuman and Harley, 1983). However, this discrepancy may be caused by the selective enhancement of long-term, but not short-term, plasticity by NA *in vivo*. Stimulation of the LC potentiates EPSPs 24 hours, but not 3 hours, later (Walling and Harley, 2004). Similarly, activation of the basolateral amygdala causes a β-adrenergic receptor-mediated increase in LTP maintenance in the dentate gyrus (Frey et al., 2001). This late-phase potentiation is dependent on new protein synthesis (Frey et al., 2001; Walling and Harley, 2004), a key characteristic of stable forms of LTP and long-term memory (Davis and Squire, 1984; Huang et al., 1996; Squire and Barondes, 1970).

Area CA3

LTP in area CA3 is β-adrenergic receptor-dependent. Blockade of these receptors during HFS prevents early and late phases of LTP (Huang and Kandel, 1996). Correspondingly, application of NA or β-adrenergic agonists elicits a frequency-dependent increase in the magnitude, duration and induction probability of LTP (Hopkins and Johnston, 1984; Hopkins and Johnston, 1988). During low-frequency stimulation (LFS), activation of β-adrenergic receptors has little effect on synaptic strength at mossy fibre synapses (Hopkins and Johnston, 1984; Hopkins and Johnston, 1988). Similarly, pairing

β-adrenergic receptor activation with LFS at associational-commissural CA3 synapses does not induce plasticity (Moody et al., 1998).

However, applying a β-adrenergic agonist during weak tetanus increases the likelihood for LTP induction, and increases LTP expression. Electrical stimuli that are subthreshold for LTP induction are able to generate LTP when β-adrenergic receptors are activated. β-Adrenergic receptors also elicit late-phase LTP when paired with stimulation protocols that normally induce early-phase LTP (Huang and Kandel, 1996). Therefore β-adrenergic receptor activation can modulate properties of LTP, but cannot increase synaptic strength without concurrent high frequency stimulation.

The mechanism for this modulation of LTP is thought to be presynaptic (Huang and Kandel, 1996), consistent with studies demonstrating that HFS and forskolin-dependent LTP are presynaptically mediated in this subregion (Huang et al., 1994; Reid et al., 2004; Zalutsky and Nicoll, 1990). In this manner, endogenous actions of NA on mossy fibre presynaptic terminals could increase excitatory transmitter release and enhance initial expression of LTP (Huang and Kandel, 1996).

Area CA1

Unlike other hippocampal subregions, β-adrenergic receptors in area CA1 are not required for the induction of LTP by HFS (Dunwiddie et al., 1982; Murchison et al., 2004; Sarvey et al., 1989; Swanson-Park et al., 1999). Application of β-adrenergic agonists to area CA1 does not persistently alter basal synaptic strength (Thomas et al., 1996). These studies suggest that the role of β-adrenergic receptors in this hippocampal subregion is distinct from their role in the dentate gyrus and area CA3.

Conversely, β-adrenergic receptor activation importantly modulates the effects of LFS on synaptic strength. Long trains of LFS appear to activate protein phosphatases

(Norman et al., 2000; O'Dell and Kandel, 1994; Thomas et al., 1996) that oppose LTP induction and cause a transient decrease in synaptic strength (Woo and Nguyen, 2002). Pairing this LFS stimulation with activation of β-adrenergic receptors overcomes the phosphatase-mediated inhibition and permits induction of LTP (Thomas et al., 1996; Winder et al., 1999). Induction of this LTP is dependent on the β1-adrenergic receptor, and requires PKA and ERK signaling cascades (Giovannini et al., 2001; Thomas et al., 1996; Winder et al., 1999).

Bursts of action potentials known as "complex spikes" could enhance LTP induction during β-adrenergic receptor activation. *In vivo*, CA1 pyramidal cells fire complex spikes that are critical to plasticity (Kandel and Spencer, 1961; Lisman, 1997). Complex spikes also facilitate the generation of LTP by particular patterns of LFS *in vitro* (Thomas et al., 1998). β-Adrenergic receptor activation during LFS doubles the amplitude of these complex spikes in a PKA-dependent manner (Hoffman and Johnston, 1999), potentially amplifying the postsynaptic depolarization elicited by weak electrical stimulation and permitting induction of LTP. Furthermore, β-adrenergic receptor-mediated enhancement of LTP induction is observed during theta-burst stimulation, a protocol that mimics the *in vivo* firing pattern of pyramidal cells seen during spatial exploration in rodents (Otto et al., 1991; Swanson-Park et al., 1999).

Neuromodulators such as NA can influence the 'state' of a synapse, altering its response to future stimulation. This plasticity process is known as "metaplasticity" (Abraham, 1999). Activation of naïve synapses with certain stimulation protocols induces LTP, and also initiates a metaplastic process that prevents further LTP induction by the same pattern of stimulation (Abraham and Tate, 1997; Frey et al., 1995). Application of a β-adrenergic agonist can inhibit this metaplastic process, and permit subsequent induction of LTP at previously activated synapses (Moody et al., 1999). Concurrent activation of α- and β-adrenergic receptors also prevents the activity-dependent reversal, or depotentiation,

of LTP (Katsuki et al., 1997). Furthermore, the time window for associative LTP is enhanced by activation of β-adrenergic receptors (Lin et al., 2003b). Taken together, these studies suggest that NA acting through β-adrenergic receptors can also engage metaplastic processes to decrease the threshold for future induction of LTP.

Subiculum

Few studies have examined plasticity in the CA1-subiculum pathway (O'Mara et al., 2000). Activation of β-adrenergic receptors in this subregion facilitates the induction of LTP by electrical stimulation commonly used to induce LTD in other hippocampal subregions, and prevents induction of LTP by higher frequency electrical stimulation (Huang and Kandel, 2005). This frequency dependence may reflect high endogenous stimulation of β-adrenergic receptors by LTP-inducing stimuli. Exogenous application of β-adrenergic agonists therefore saturates this β-adrenergic receptor-dependent mechanism and inhibits LTP induction (Huang and Kandel, 2005). Shifts in β-adrenergic neuromodulation of plasticity in the hippocampus could reflect differences in subregional functions and the processing of information within the hippocampal trisynaptic circuit.

## 6. Cognitive Noradrenergic Neuromodulation

Noradrenergic modulation within the central nervous system contributes to effective processing of salient information (Berridge and Waterhouse, 2003). Activation of α- and β-adrenergic receptors in the basal forebrain is critical for waking behaviour. Blockade of these receptors decreases behavioural and electrophysiologic indices of attention and arousal (Stone and Quartermain, 1999).

Similarly, NA is implicated in enhancing the processing of sensory information. Application of NA decreases spontaneous discharge of sensory neurons, while preserving or increasing responses to specific synaptic input (Foote et al., 1975; Moises et al., 1981).

Within cellular networks, NA can potently augment the signal-to-noise ratio for processing of stimuli (Berridge and Waterhouse, 2003). Behaviourally, this increase in signal-to-noise ratio could enhance attention and vigilance, especially in stimulus-rich environments that may be distracting (Mehta, 2001).

Studies examining activation of LC neurons suggest that the cardinal function of noradrenergic neuromodulation is to facilitate the rapid reorganization of neural networks in response to contexts that require cognitive and behavioural shifts (Bouret and Sara, 2005). This rearrangement of functional connectivity between neurons in a network resembles the alterations in synaptic strength that are thought to underlie learning and memory. Correspondingly, NA plays a central role in memory processing in various brain regions (Brown and Silva, 2004; Harley, 2004; McGaugh, 2002).

a) Declarative Memory

The hippocampus is critical for creating new memories of facts, events, and places that can be consciously recalled and expressed explicitly (Eichenbaum and Cohen, 2001; Zola-Morgan et al., 1986). This declarative memory is often assessed in laboratory animals by screening their performance on spatial and associative memory tasks. NA has been found to enhance memory for a variety of hippocampus-dependent tasks.

*ι. α-adrenergic receptors*

α1-Adrenergic receptors make small, but measurable, contributions to hippocampal memory (Introini-Collison et al., 1992; Sirvio and MacDonald, 1999). Application of α1-adrenergic receptor agonists subtly facilitates acquisition of the spatial water maze task in rats (Puumala et al., 1998; Riekkinen et al., 1997). However, this enhancement is possibly due to an alteration in strategy rather than increased memory (Sirvio and MacDonald, 1999). Furthermore, effects of α1-adrenergic antagonists are often observable only in the presence of other neuromodulatory or neurotransmitter deficits (Puumala et al., 1996;

Riekkinen et al., 1996). On a cellular level, the ability of α1-adrenergic receptor activation to facilitate increases in cAMP downstream of β-adrenergic receptors could explain these milder, synergistic effects on memory function.

Conversely, up-regulation of α2-adrenergic receptors actually decreases performance on spatial memory tasks. Constitutive activity of α2-adrenergic receptors in the hippocampus produces deficits in spatial navigation in the water maze (Bjorklund et al., 1998). It is possible that increasing α2-adrenergic receptor–mediated presynaptic inhibition interferes with hippocampal memory processes.

*ii. β-adrenergic receptors*

Activation of β-adrenergic receptors accounts for the majority of NA-dependent memory effects. Injection of β-adrenergic receptor antagonists into the hippocampus inhibits memory for spatial water maze tasks (Ji et al., 2003a) and contextual fear conditioning (Ji et al., 2003b). Similarly, administration of these antagonists in acute or chronic doses inhibits spatial and associational memory (Cahill et al., 2000; Czech et al., 2000; Sara et al., 1999). Intracellular signaling via the cAMP-PKA cascade in the hippocampus may mediate these responses downstream of the β-adrenergic receptor (Barros et al., 1999; Bevilaqua et al., 1997).

Signaling through β-adrenergic receptors affects specific temporal phases of memory. These receptors are implicated in long-term (LTM), rather than short-term (STM), memory processes. NA injected into area CA1 of the hippocampus selectively enhances LTM without altering STM (Izquierdo et al., 1998), an effect that is mimicked by infusion of drugs that stimulate PKA (Barros et al., 1999). Post-conditioning β-adrenergic receptor blockade also impairs LTM for contextual fear conditioning (Ji et al., 2003b). Furthermore, β-adrenergic antagonists inhibit long-term memory retention for an associative task when applied two hours after training, suggesting a role in a late-phase of memory storage (Sara

et al., 1999). Based on these temporal requirements for β-adrenergic receptor activation, a role for the noradrenergic system in memory consolidation has been suggested (Izquierdo and Medina, 1997; McGaugh, 2000). Some studies, however, did not find evidence for β-adrenergic receptor-dependent memory consolidation and suggest that noradrenergic neuromodulation is not generally necessary for memory consolidation (Lee et al., 2001; Thomas and Palmiter, 1997). Interpretation of these behavioural data is complicated by the fact that extended training time was required for some tasks, during which memory may be repeatedly acquired, consolidated, retrieved, and reconsolidated (Murchison et al., 2004).

Because time courses for temporal phases of memory can vary depending on the behavioural task used, memory processing is often defined by acquisition, consolidation, and retrieval stages. These stages may selectively recruit specific brain regions and molecular mechanisms, allowing for better isolation of different memory processes (Abel and Lattal, 2001). There is some evidence to suggest that NA acts through β-adrenergic receptors in the hippocampus to mediate retrieval of memories (Barros et al., 2001; Devauges and Sara, 1991; Murchison et al., 2004). Stimulation of the LC or injection of NA into the hippocampus promotes retrieval of memory for food-motivated maze and inhibitory avoidance tasks (Barros et al., 2001; Devauges and Sara, 1991).

However, these studies did not demonstrate that β-adrenergic receptor activation was *necessary* for retrieval. Knockout mice that lack endogenous NA and adrenaline have been used to address this issue (Murchison et al., 2004). These mice display impaired long-term contextual fear memory that can be rescued by pre-testing, but not by pre-training, restoration of NA levels. These results were shown to depend on β-, but not α-adrenergic receptor activation, and can be replicated in rats and for other hippocampus-dependent memory tasks (spatial water maze) (Murchison et al., 2004). Therefore, in the absence of NA, memories are acquired and consolidated, but cannot be retrieved. However, these conditional knockout mice lack NA from birth. Because restoration of NA in the mice

during both training and testing rescues contextual fear conditioning, this memory deficit is probably not due to a developmental abnormality. Neuromodulatory compensation in other brain systems remains unexplored.

β-Adrenergic receptors are also implicated in post-retrieval memory processing. Interestingly, the β-adrenergic receptor-dependency of retrieval is time-limited. One week post-training, memory retrieval is restored in the NA-deficient mice (Murchison et al., 2004). This may reflect the transfer of memory from the hippocampus to cortical storage sites (Bontempi et al., 1999; Frankland et al., 2001; Ross and Eichenbaum, 2006). Furthermore, blockade of β-adrenergic receptors after the reactivation of a memory induces amnesia when this memory is tested at a later time point (Przybyslawski et al., 1999). β-Adrenergic signaling is also required for the extinction of memory, a process which allows new associations to be made about previously-experienced stimuli (Ouyang and Thomas, 2005). Therefore, β-adrenergic receptors may contribute to the complex, and as yet poorly understood, interactions that ultimately determine the relative strength of stored memories.

b) Emotional Memory – Hippocampus-Amygdala Interactions

Stress hormones, including NA and adrenaline, are strongly implicated in emotional memory storage. Systemic injection of adrenaline to mimic emotional stimuli increases retention for inhibitory avoidance tasks (Gold et al., 1977). Subsequent studies indicated that peripheral adrenaline mediates this memory enhancement by activating β-adrenergic receptors on vagal afferents in the nucleus of the solitary tract (NST). Noradrenergic projections from the NST innervate forebrain structures and brainstem nuclei, including the amygdala (Van Bockstaele et al., 1998; Williams and McGaugh, 1993; Williams et al., 1998).

Emotional stimuli also activate the hypothalamic-pituitary-adrenocortical axis, releasing glucocorticoids into peripheral circulation. Glucocorticoids influence numerous memory processes, including memory consolidation (de Kloet et al., 1999; McEwen, 2000;

Roozendaal et al., 2002). The amygdala is a crucial locus of action for glucocorticoid-mediated memory enhancements (Roozendaal and McGaugh, 1996). Activation of amygdalar glucocorticoid receptors elicits behavioural responses by increasing the potency of noradrenergic signaling via interactions of G-proteins (Roozendaal, 2002).

The facilitatory effects of emotional arousal on memory can therefore be mostly attributed to signaling downstream of noradrenergic receptors in the amygdala. Although activation of α1- and β-adrenergic receptors in the amygdala contributes to memory enhancement, the α1-adrenergic receptor-mediated response requires co-activation of β-adrenergic receptors (Ferry et al., 1999a; Ferry et al., 1999b; Hatfield and McGaugh, 1999). β-Adrenergic antagonists infused directly into the amygdala inhibit memory enhancements caused by peripheral injections of adrenaline (Liang et al., 1986) or glucocorticoids (Quirarte et al., 1997). Furthermore, studies of human subjects demonstrate that β-adrenergic receptor antagonists selectively prevent the increased retention of memory caused by exposure to emotional stimuli, without affecting memory for neutral stimuli (Cahill et al., 1994; van Stegeren et al., 1998). Stimulation of the noradrenergic neuromodulatory system can likewise enhance emotional memory (O'Carroll et al., 1999).

However, behavioural studies in animals indicate that the amygdala mediates effects on memory by modulating responses in other brain regions. Injection of amphetamine into the hippocampus or caudate nucleus selectively enhances retention for hippocampus- or caudate-dependent memory tasks, respectively. Activation of the amygdala with amphetamine, however, facilitates memory for both hippocampus- and caudate-dependent tasks. Subsequent inactivation of the amygdala prior to retrieval does not prevent expression of the memory enhancements (McGaugh et al., 1996). Thus, the amygdala may promiscuously modulate memory in several brain regions, without functioning as a site for long-term memory storage.

In particular, reciprocal interactions between the amygdala and the hippocampus may be responsible for the facilitation of declarative memory pertaining to emotional material (McGaugh et al., 1996). This facilitation can be assessed in humans using functional neuroimaging methods. Increased and more highly correlated activity in the amygdala and MTL during encoding of stimuli predicts enhanced memory for emotional stimuli (Dolcos et al., 2004; Kensinger and Corkin, 2004). In patients with epilepsy or MTL sclerosis, the degree of pathology in the hippocampus determines memory for both emotional and neutral material. Pathology of the amygdala selectively impairs memory for emotional materials. Furthermore, encoding activity in the hippocampus for emotional materials is dependent on the degree of amygdalar pathology (Richardson et al., 2004). These results suggest that the hippocampus and amygdala function cooperatively to acquire emotional memory. Anatomical pathways connecting the amygdala and hippocampus likely convey important information between these brain structures (Pitkanen et al., 2000).

The retrieval of emotional memory after extended retention intervals is also correlated with increased activity in the amygdala and hippocampus. One year after an encoding session, retention was greater for emotional than neutral stimuli. Successful retrieval of the emotional items augmented activity in the hippocampus and amygdala relative to retrieval of neutral items (Dolcos et al., 2005). The increased signaling efficacy responsible for enhancement of emotional memory shortly after encoding is therefore retained over time, and is operational during memory retrieval.

NA acting through β-adrenergic receptors plays an important role in interactions between the amygdala and hippocampus. Enhancements in episodic memory for emotional words are blocked by application of β-adrenergic receptor antagonists (Strange et al., 2003). Furthermore, successful encoding-evoked activation in the amygdala for emotional items is inhibited by β-adrenergic receptor blockade. Recognition of these same emotional items triggers activity in the hippocampus only if β-adrenergic receptors are functional during

encoding (Strange and Dolan, 2004). Activation of β-adrenergic receptors may therefore mediate the amygdala-dependent modulation of hippocampal memory for emotional stimuli (**Figure 1.9**).

## 7. Objectives of the Present Thesis

Neuromodulators strongly influence the mammalian brain on molecular, cellular, and behavioural levels. They can affect biophysical properties of ion channels, firing of neural networks, and higher cognitive processes. Understanding the contribution of neuromodulation to neural processes could shed light onto the relationship between neurons and cognition. As such, the primary aim of my research is to investigate effects of the neuromodulator NA on synaptic plasticity, a phenomenon hypothesized to underlie memory storage.

The initial objective of this thesis was to characterize the role of NA in long-term synaptic plasticity in area CA1 of the hippocampus. Next, I aimed to determine whether selective activation of β-adrenergic receptors could facilitate the maintenance of long-term synaptic plasticity, and if so, elucidate the characteristics of this LTP. The third objective was to determine the signaling pathways and molecules activated downstream of β-adrenergic receptors, with primary focus on engagement of protein synthesis. Specifically, I addressed the following questions:

1. Does NA facilitate the stability of LTP in area CA1?
2. What receptor subtype(s) are responsible for the role of NA in LTP maintenance?
3. What are the characteristics and molecular requirements of β-adrenergic receptor-dependent enhancement of LTP maintenance?
4. Does activation of β-adrenergic receptors engage protein synthesis to stabilize LTP, and if so, how?
5. What is the role of the recently discovered exchange protein activated by cAMP (Epac) on the stability of synaptic plasticity?

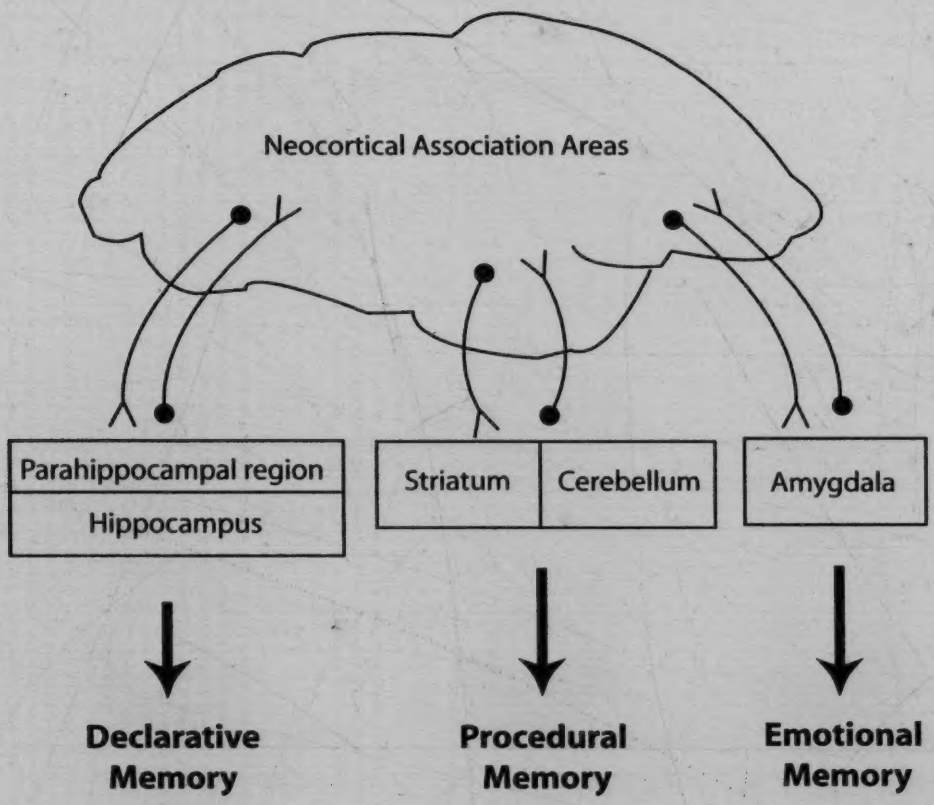
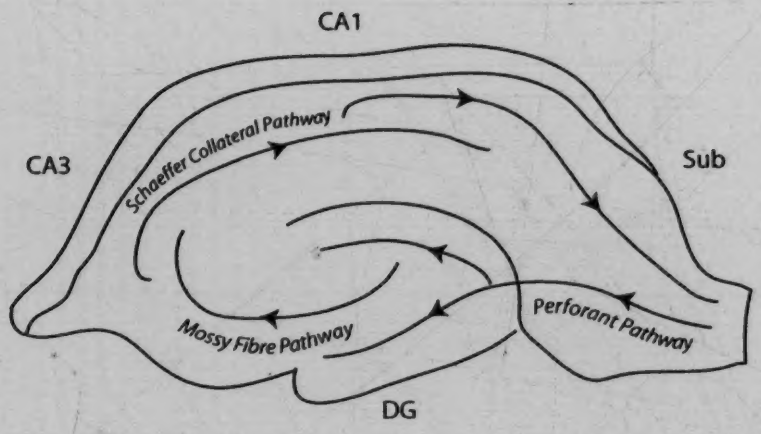



Figure 1.1: Three major memory systems and their associated brain regions (adapted from Eichenbaum, 2002).

A.




B.

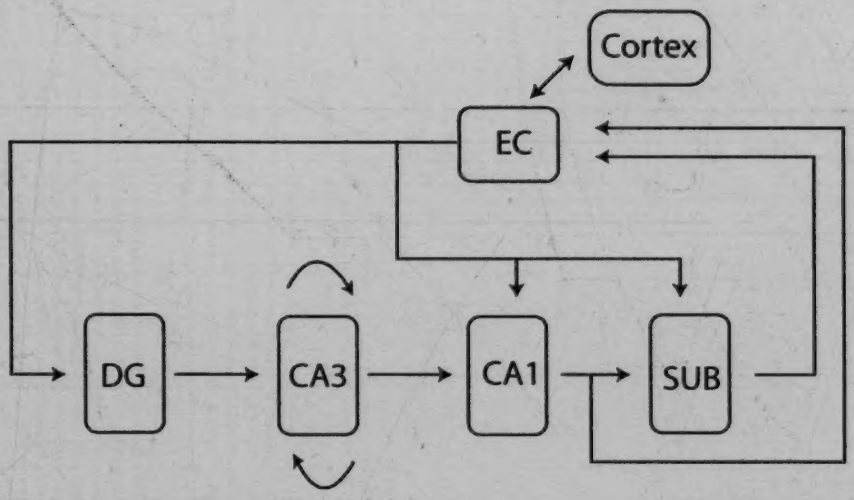



Figure 1.2: Hippocampal circuitry. (A) The trisynaptic pathway of the hippocampus is composed of: i) the perforant pathway, projecting from the entorhinal cortex (EC) to the dentate gyrus (DG); ii) the mossy fibre pathway, projecting from the DG to area CA3; iii) the Schaeffer collateral pathway, projecting from area CA3 to area CA1. CA1 pyramidal neurons send efferents through the subiculum (SUB) and back to the entorhinal cortex (EC). (B) Schematic diagram illustrating the 'long' and 'short' hippocampal pathways (adapted from Eichenbaum and Cohen, 2001).

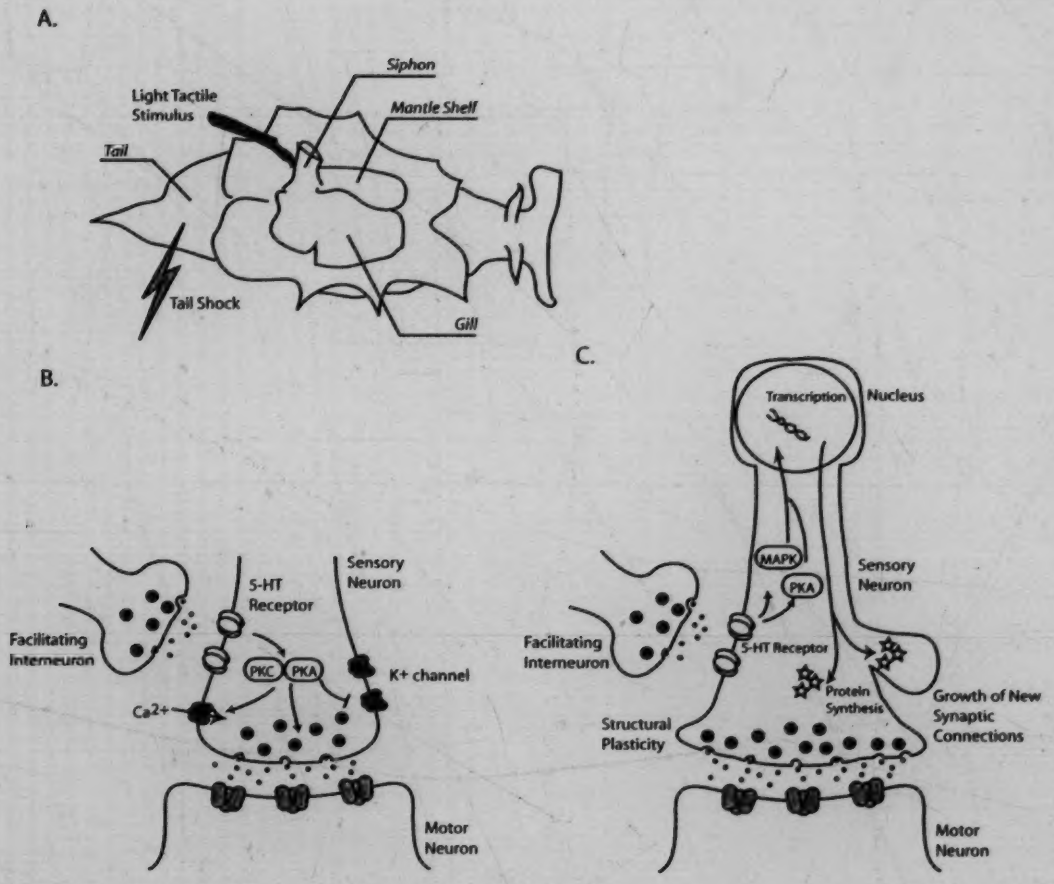



Figure 1.3: Sensitization in Aplysia. (A) Aplysia anatomy (dorsal view). An electrical shock to the tail enhances the amplitude and duration of gill withdrawal elicited by a subsequent light tactile stimulus. (B) Cellular mechanisms of short-term sensitization. A single tail shock causes release of serotonin (5-HT) from facilitating interneurons. 5-HT activates PKA and PKC signaling cascades, which (1) mobilize neurotransmitter vesicles for release, (2) decrease potassium conductance to prolong action potentials, (3) enhance $Ca^{2+}$ conductance through voltage-gated calcium channels, and (4) facilitate $Ca^{2+}$-dependent transmitter release. (C) Cellular mechanisms of long-term sensitization. Repeated tail shocks cause increased 5-HT release, and recruit PKA and MAPK signaling cascades. These kinases can translocate to the nucleus and induce transcription and protein synthesis. Gene products contribute to structural plasticity and growth of new synaptic connections (adapted from Kandel et al., 2000).

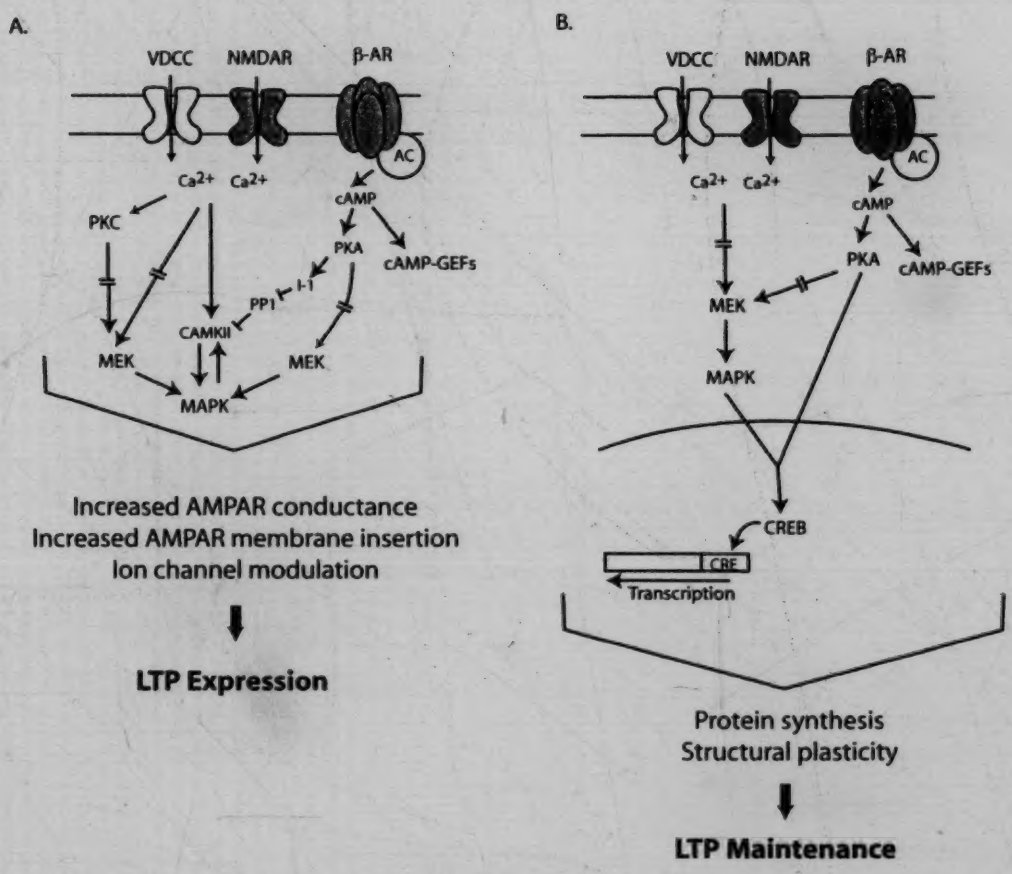



Figure 1.4: Key signaling pathways contributing to LTP expression and maintenance. (A) Activation of NMDA receptors, VDCCs, and Gs protein-coupled receptors (such as the β-adrenergic receptor) engages CaMKII, PKA, PKC, and MAPK signaling cascades. Interaction between these cascades results in increased synaptic efficacy and expression of LTP. (B) PKA and MAPK can also translocate to the nucleus and stimulate transcription via activation of CREB. Subsequent protein synthesis and structural plasticity underlie maintenance of LTP.

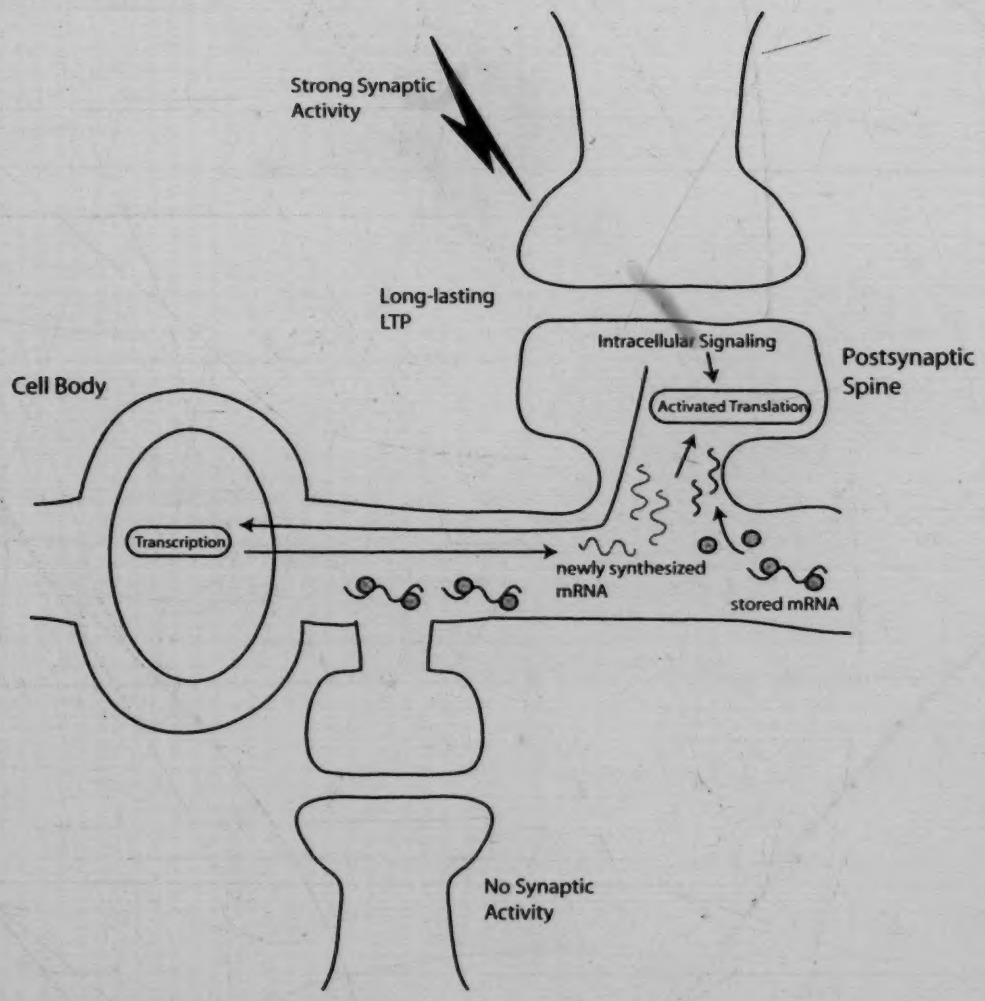



Figure 1.5: Simplified schematic of macromolecular synthesis during L-LTP. Strong synaptic stimulation initiates translation of stored mRNAs located in the dendrites. Concurrently, a signal is carried to the nucleus to initiate transcription. Newly synthesized mRNAs are distributed throughout the neuron. The translation of stored and newly synthesized mRNAs at the active synapse in a spatially restricted manner is hypothesized to result in production of plasticity proteins and long-term synaptic modifications only at the site of stimulation.

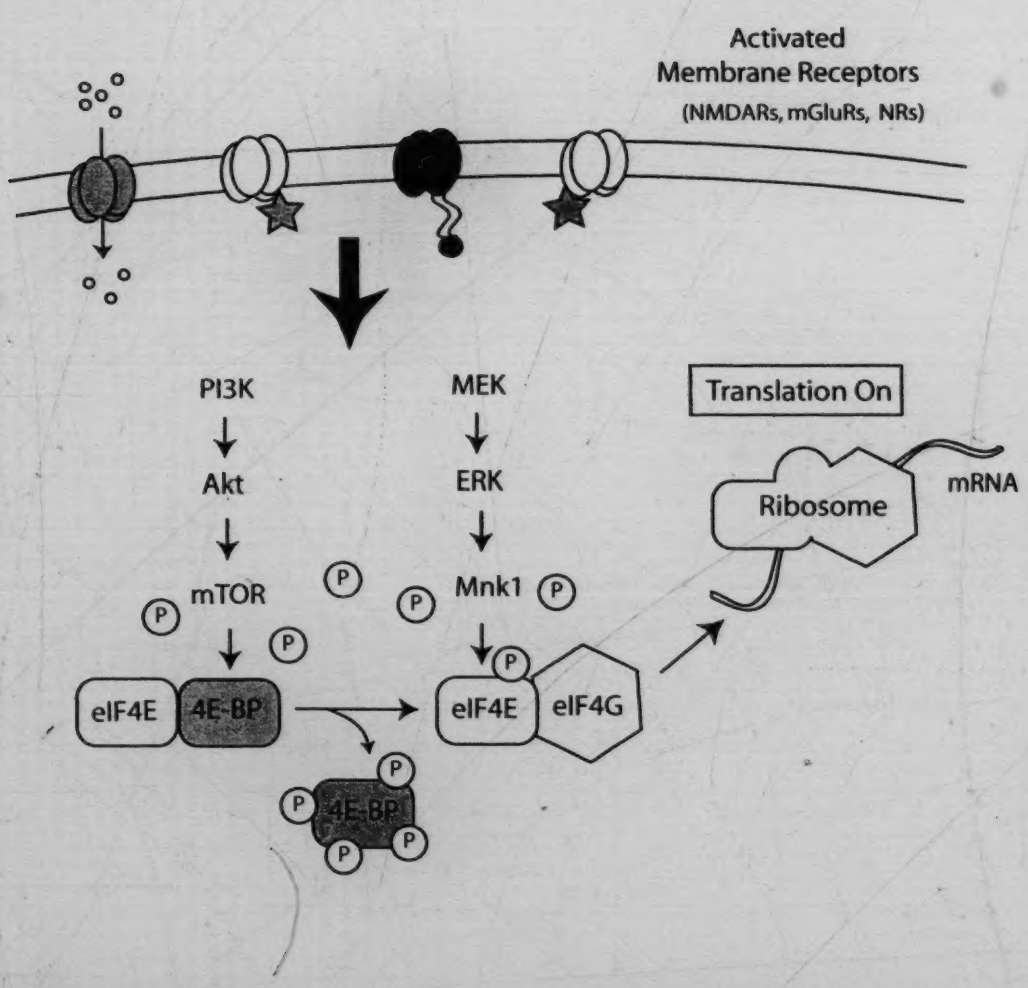



Figure 1.6: Signaling pathways involved in regulation of translation initiation. Engagement of the PI3K-mTOR pathway causes mTOR to phosphorylate the binding protein 4E-BP, which subsequently dissociates from the translation initiation factor eIF4E. eIF4E then binds to eIF4G to promote mRNA binding and formation of the initiation complex. Phosphorylation of eIF4E by the ERK-activated kinase Mnk1 is associated with enhanced translation. In this manner, PI3K-mTOR and ERK pathways can critically regulate protein synthesis-dependent synaptic plasticity (adapted from (Klann and Dever, 2004).

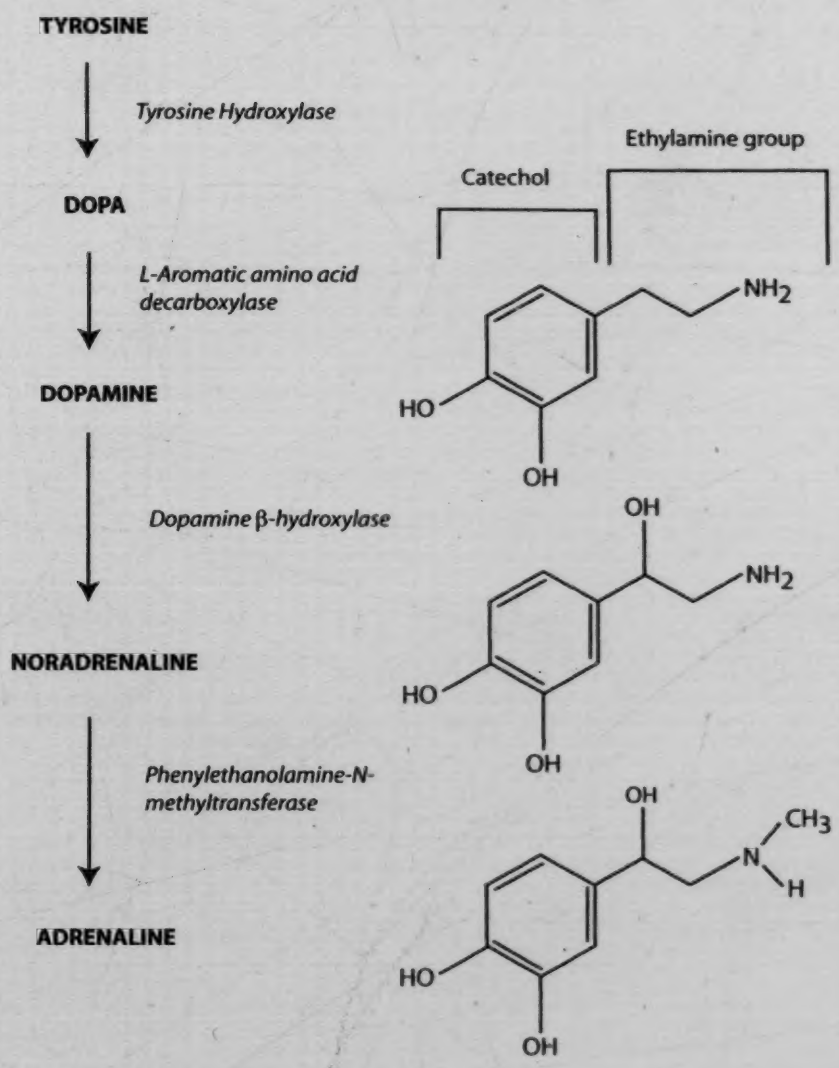



Figure 1.7: Primary synthesis pathway for dopamine, noradrenaline, and adrenaline.

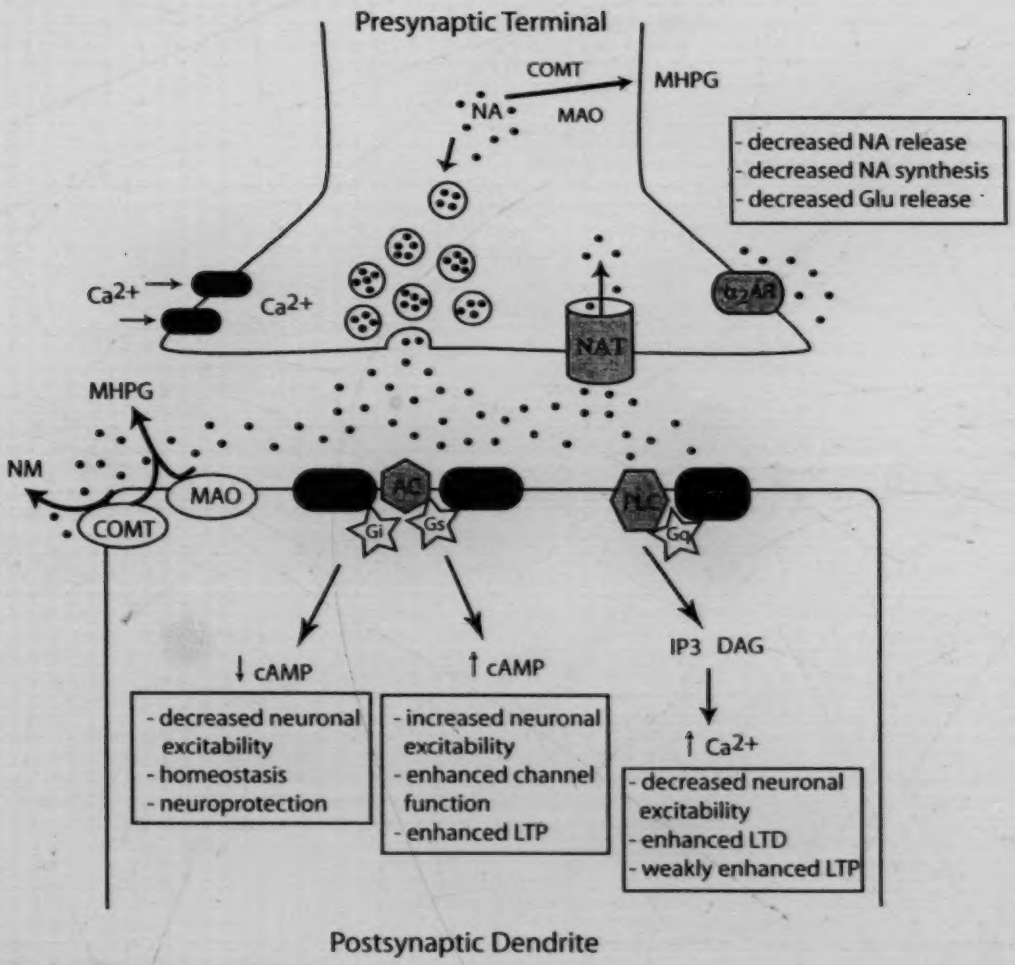



Figure 1.8: Schematic of a noradrenergic nerve terminal. NA is released into the synaptic cleft and interacts with α1-, α2- and β–adrenergic receptors. Presynaptic α2-adrenergic receptors regulate the synthesis and release of NA, whereas postsynaptic receptors mediate various cellular responses. Action of NA is terminated by reuptake through NAT, and metabolic degradation by MAO and COMT. VDCC: voltage-dependent calcium channel; AR: adrenergic receptor; NAT: plasma membrane noradrenaline transporter; MHPG: 3-methoxy-4-hydroxy phenethylene glychol; NM: normetanephrine; AC: adenylyl cyclase (adapted from Cooper et al., 2003).

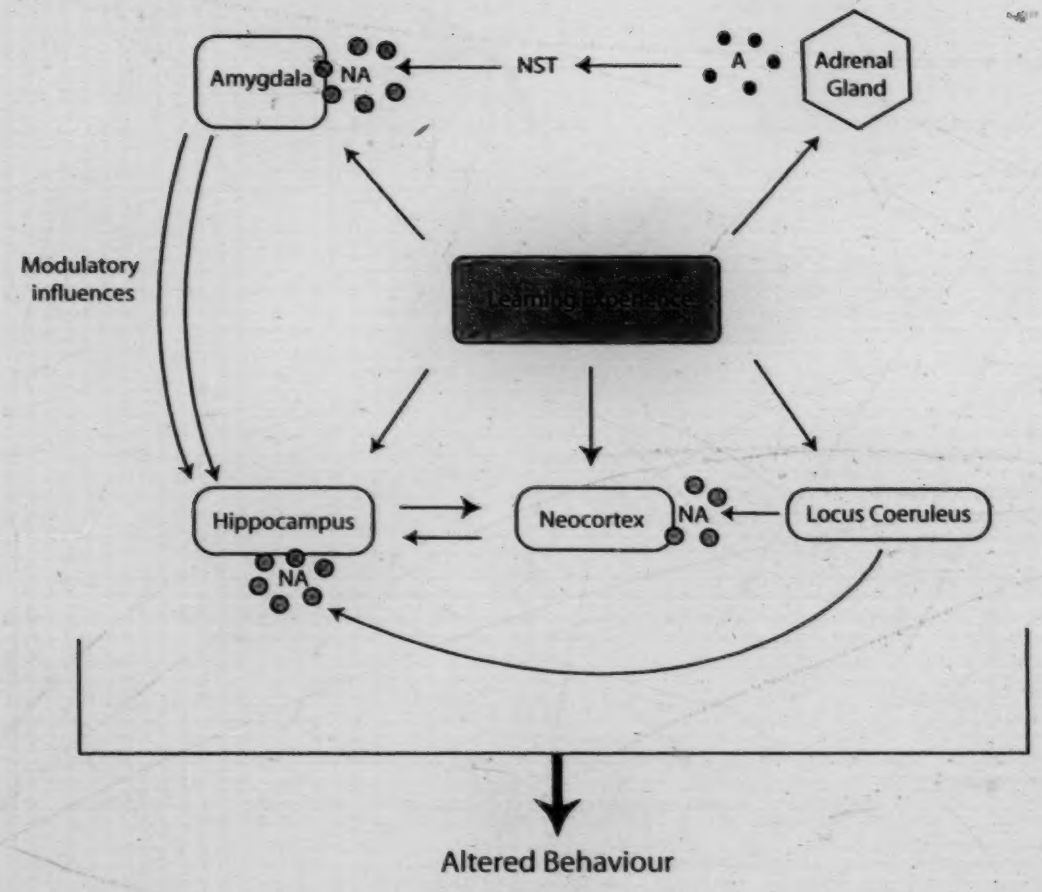



Figure 1.9: Noradrenergic modulation of hippocampus-dependent memory. A learning experience initiates memory formation in the hippocampus, which can be modulated by NA release from the locus coeruleus. The experience also induces release of stress hormones from the adrenal gland that act on the nucleus of the solitary tract (NST) to induce NA release in the amygdala. The amygadala subsequently influences memory consolidation in the hippocampus via modulatory pathways. Interaction between the hippocampus and neocortex establishes long-term consolidation of memory (adapted from McGaugh, 2000).

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# CHAPTER II

## General Methodology

## 1. Animals

### a) C57BL/6 Mice

Because mice within an inbred strain are genetically homogeneous, they are commonly used in research applications. C57BL/6 is one widely studied strain that displays robust hippocampal synaptic plasticity and reproducible performance on several tests of behavioural learning and memory (Wehner and Silva, 1996; Owen et al., 1997; Schimanski and Nguyen, 2004). Female C57BL/6 mice obtained from Charles River, Canada, were used for all experiments unless otherwise stated. Mice were sacrificed at 8-13 weeks of age for electrophysiology. Housing and experimental procedures followed guidelines approved by the Canadian Council on Animal Care (CCAC).

### b) R(AB) Mice

R(AB) transgenic mice were generously supplied by my collaborator, Dr. Ted Abel (University of Pennsylvania). The R(AB) transgene is a dominant negative form of the RIα regulatory subunit of PKA (Clegg et al., 1987; Abel et al., 1997). This transgene was placed under control of the CaMKIIα promoter to drive expression of R(AB) in the postnatal neocortex, olfactory bulb, hippocampus, striatum, and amygdala. As a result, basal PKA activity is decreased by approximately 10-fold in transgenic mice relative to wildtype littermates. When PKA is stimulated with 5 μM cAMP, a significant deficit in PKA activity is also observed (Young et al., 2006). R(AB) mice were originally derived from two independent lines that were characterized for R(AB) transgene expression, hippocampal PKA activity, hippocampal synaptic physiology, and behavioural memory. BL6CBAF2/J founder animals were backcrossed to C57BL/6J mice to obtain these lines.

Currently, the R(AB) colony is maintained in a hemizygous state at N10-N13 on a C57BL/6J background. Genotyping was performed using tail DNA analyzed by Southern blot with a transgene specific probe. Male and female R(AB) mice, aged 6-10 months, were used for all experiments. Age-matched wildtype littermates were used as experimental controls, with experimenters blind to genotype. Animals were maintained according to guidelines of both the Institutional Animal Care and Use Committee (IACUC) and Canadian Council on Animal Care (CCAC).

**2. *In vitro* Electrophysiology**

a) Hippocampal Slice Preparation

Mice were sacrificed by cervical dislocation and decapitation. The intact brain was quickly removed and placed in a beaker of ice-cold artificial cerebrospinal fluid (ACSF). The ACSF contained (in mM): 125 NaCl, 4.4 KCl, 1.5 $MgSO_4$, 1.0 $NaH_2PO_4$, 26 $NaHCO_3$, 10 glucose, 2.5 $CaCl_2$. After approximately one minute of cooling, the brain was hemisected down the longitudinal fissure. A hippocampus from one cerebral hemisphere was isolated from surrounding tissue and placed on a manual tissue chopper (Stoelting, Wood Dale, IL, USA). Transverse hippocampal slices 400 μm thick were then sliced and transferred to an interface recording chamber (Fine Science Tools, Vancouver, Canada). The slicing procedure was repeated for the hippocampus in the other cerebral hemisphere. Slices were maintained at 28°C. ACSF, aerated with carbogen gas (95% oxygen, 5% carbon dioxide), was continuously perfused through the interface chamber at a rate of approximately 1 mL/min. Slices were allowed to recover for at least one hour prior to commencement of recording.

b) fEPSP Recordings

Extracellular field excitatory post-synaptic potentials (fEPSPs) were elicited from area CA1 of the hippocampal slice. A bipolar nickel-chromium stimulating electrode (diameter 130 μm; AM Systems, Carlsborg, WA) was used to stimulate Schaeffer collateral fibres in stratum radiatum of CA1. Recordings from stratum radiatum were obtained using a glass microelectrode (resistance 2-4 MΩ) filled with ACSF. Consistent electrode placement was maintained using the pyramidal cell body layer of CA1 as a visual landmark. Baseline fEPSP responses were elicited at 40% of maximal fEPSP amplitude with a pulse width of 0.08 ms. These baseline 'test' responses were evoked once per minute.

Two-pathway recording was performed using two stimulating electrodes placed on either side of a recording electrode in stratum radiatum. This experimental setup allowed stimulation of two separate groups of Schaeffer collateral fibres converging onto the same postsynaptic population of neurons. During the course of an experiment, the second independent pathway was often used to monitor potential effects of applied drugs on basal synaptic transmission. The independence of the pathways was verified by the absence of inter-pathway paired pulse facilitation when successive stimulation was applied through the two electrodes at 40, 50, 75, 100, 150 and 200 ms intervals (Young and Nguyen, 2005; Young et al., 2006).

Input-output (I/O) data were generated by varying the stimulus intensity of 7 stimuli applied to the Schaeffer collateral pathway. Paired-pulse facilitation (PPF) was assessed by giving paired pulses separated by 50, 100, 150, and 200 ms intervals. Various electrical stimulation protocols were used to alter synaptic strength. High

frequency stimulation (HFS) protocols included one 1 s train of 100 Hz stimulation (at 'test' strength) and four 1 s trains of 100 Hz stimulation (at 'test' strength) spaced at 5 min intervals. Depotentiation was induced by giving low frequency stimulation (LFS) at 5 Hz for 3 min following LTP induction. Theta-train stimulation consisted of LFS at 5 Hz for 3 min.

All evoked fEPSPs were measured by an intracellular amplifier and low pass filtered at 2 kHz. Responses were digitized at a rate of 20 kHz by the Digidata 1200 acquisition system and analyzed with pClamp 7 software (Axon Instruments Inc., Union City, CA, USA).

c) Drug Preparation and Application

Several drugs were used to probe the mechanisms of synaptic plasticity. Drugs were dissolved in appropriate solvents to make concentrated stock solutions. Aliquots of the stock solutions were stored at -20°C. Immediately prior to experimentation, aliquots were thawed, vortexed thoroughly, and diluted in ACSF to the desired final drug concentration. Because catecholamines undergo oxidation in the presence of oxygen or other oxidants, and at alkaline pH, fresh stock solutions of isoproterenol and/or noradrenaline were prepared daily.

Drugs were bath applied to hippocampal slices (1-2 mL/min). When drugs were prepared in dimethylsulfoxide (DMSO), the final concentration of DMSO in the bath did not exceed 0.1%. At this concentration, DMSO does not affect basal synaptic transmission (data not shown). Because many of the drugs used were light-sensitive, experiments were performed in dimmed light. Drug experiments were interleaved with

drug-free controls. **Table 2.1** describes each drug used on the basis of its physiological actions, concentration, appropriate solvent, and duration of application.

**3. Biochemistry**

a) Sample Preparation

Hippocampal slices were prepared and maintained as described above. Following pharmacological and/or electrical stimulation, slices were harvested from the interface recording chamber. An upright dissecting microscope and razor blade were used to microdissect area CA1. CA1 subregions were snap-frozen in liquid nitrogen and stored at -80°C until assayed. Four to five CA1 subregions were pooled for each experimental condition.

The pooled CA1 subregions were homogenized by sonication. The homogenization buffer (HB) contained (in mM): 10 HEPES, 1 EGTA, 1 EDTA, 10 $Na_4P_2O_7$, 150 NaCl, 50 NaF, 2 μg/mL aprotinin, 10 μg/mL leupeptin, 200 nM calyculin A, 1 μM microcystin. Samples with equivalent amounts of protein, as determined by the Bradford method (Bradford, 1976), were loaded onto 4-20% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels. Standard gel electrophoresis was then used to resolve the samples.

b) Immunoprecipitation

Sample homogenates were precleared with 50% protein A magnetic bead slurry for one hour. Sequential incubation with anti-eIF4G1 antibody (10 μg) and 50% protein A magnetic bead slurry was performed for one hour at room temperature. The samples were then subjected to a magnetic field and the supernatant was discarded.

Immunoprecipitated protein complexes were washed with fresh HB and eluted from the beads with β-mercaptoethanol before analysis by quantitative Western blotting (**Fig 2.1**).

**4. Data Analysis**

a) Analysis of Electrophysiology

The initial slope of an fEPSP is used as a measure of synaptic strength (Johnston and Wu, 1995). Digitized fEPSP recordings were analyzed offline to determine initial slopes with pClamp 7.0 software (Axon Instruments). For LTP experiments, fEPSP slopes from 20 min of stable baseline recording were averaged to obtain a baseline slope value for each experiment. All subsequent slope values were reported as percentages of this baseline slope. I/O analysis was performed by plotting presynaptic fibre volley amplitudes against the corresponding fEPSP slopes. A linear regression value was then calculated for the data. For PPF, fEPSP slopes for each pulse in a pair were measured and the slope of the second pulse was expressed as percentage facilitation of the slope of the first pulse.

All data were tested for statistical significance using Student's t-test (two-tailed) for two groups, or analysis of variance (ANOVA) with Tukey-Kramer *post hoc* tests for three or more groups (Graphpad Instat Software, San Diego, CA, USA). The Welch correction was applied when standard deviations were found to be significantly different between groups in the Student's t-test. When comparing multiple groups with significantly different standard deviations, the Kruskal-Wallis test was used, followed by Dunn's multiple comparisons *post hoc* tests. In all cases, criteria for significance was $p <$

0.05. Values shown are expressed as mean ± standard error (SEM). For electrophysiology experiments, n is equal to number of slices.

b) Quantitative Western Blotting

Quantitative analysis of protein levels was done by my collaborator, Dr. Jessica Banko (Vanderbilt University) (**Figure 2.1**). SDS-polyacrylamide gels were first electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Reversible MemCode protein stain was used to confirm equal loading and transfer. Prior to incubation with antibodies, membranes were blocked for one hour at room temperature to prevent non-specific binding. The blocking solution contained 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, and 0.24% I-block at pH 7.5 (Tropix, Bedford, MA, USA). Primary antibodies were incubated at room temperature for one hour [phospho-ERK antibody (1:5000), phospho-Mnk1 antibody (1:1000), phospho-eIF4E antibody (1:1000), phospho-4E-BP1 antibody (1:500), total eIF4E (1:1000), or eIF4G1 antibody (1:1000)]. Incubation with horseradish peroxidase-linked goat anti-rabbit IgG (1:2500 dilution) for one hour at room temperature followed. After each antibody incubation, membranes were washed extensively in Tris-buffered saline containing 50 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20 at pH 7.5.

Blots were then developed with enhanced chemiluminescence (ECL) to visualize protein bands. These bands were quantified from film exposure in the linear range for each antibody used and normalized to the MemCode membrane staining with densitometry. Statistical analysis was performed using Graphpad Prism data analysis software. As with electrophysiology, data are represented as mean ± standard error (SEM). Student's t-test was employed to assess the data, with $p \leq 0.05$ as significance

criteria. For molecular biology experiments, n is equal to the number of pooled samples for each experimental condition.

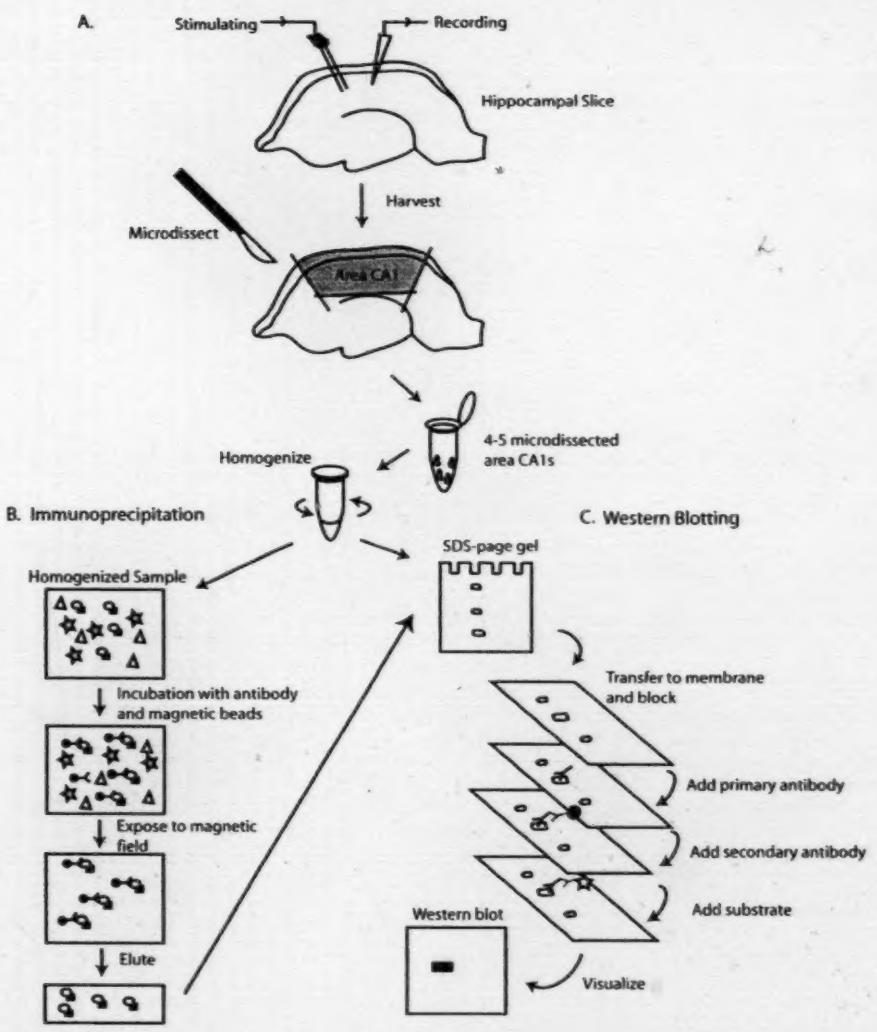


Figure 2.1: Biochemical procedures. A. Following pharmacological and/or electrical stimulation, hippocampal slices are harvested from the recording chamber and area CA1 is removed by microdissection with a razor blade. 4-5 microdissected area CA1s are pooled into a microcentrifuge tube and homogenized by sonication. B. A portion of the homogenized sample can be used for immunoprecipitation experiements. The sample is incubated with a magnetic bead slurry and an antibody of interest. Protein complexes bind to the antibody, and can be extracted from the sample by exposing the sample to a magnetic field and discarding the resulting supernatant. Immunoprecipitated complexes are eluted from the beads before analysis by Western blotting. C. Homogenized samples can also be analyzed directly by Western blot. After being resolved on an SDS-Page gel, proteins are transferred to a membrane. This membrane is first blocked, then incubated with primary and secondary antibodies. The secondary antibody is coupled to a chemiluminescent molecule that permits subsequent visualization of protein bands.

Table 2.1: Summary of Pharmalogical Agents Used in the Present Thesis

| Drug | Distributor | Physiological Action | Solvent | Concentration (μM) | Duration of Application (min) |
|---|---|---|---|---|---|
| Actinomycin D (Act-D) | Bioshop | Transcription inhibitor | DMSO | 25 | 45 |
| Anisomycin (Aniso) | Sigma | Translation inhibitor | DMSO | 25 | 45 |
| Emetine | Sigma | Translation inhibitor | Distilled water | 20 | 45 |
| Isoproterenol (ISO) | Sigma | β-adrenoceptor agonist | Distilled water | 1 | 15 |
| KT5720 | Sigma | PKA inhibitor | DMSO | 1 | 45 |
| Noradrenaline (NA) | Sigma | Adrenergic neurotransmitter | Distilled water | 10 | 15 |
| Okadaic Acid (OA) | Sigma | PP1/2A inhibitor | Distilled water | 1 | 90 |
| 8-pCPT-2'-O-Me-cAMP (8-pCPT) | Axxora | Epac agonist | DMSO | 100 | 15 |
| PD98059 | Bioshop | MEK inhibitor | DMSO | 50 | 55 |
| Prazosin | Sigma | α-1 adrenoceptor antagonist | Ethanol | 10 | 45 |
| Propranolol | Sigma | β-adrenoceptor antagonist | Distilled water | 50 | 55 |
| Rapamycin (Rap) | Bioshop | mTOR inhibitor | DMSO | 1 | 45 |
| Rp-cAMPs (Rp) | Sigma | PKA inhibitor | DMSO | 60 | 45 |
| SCH23390 | Sigma | D1 dopamine receptor antagonist | Distilled water | 1 | 45 |
| U0126 | Bioshop | MEK1, 2 inhibitor | DMSO | 20 | 45 |
| Yohimbine | Sigma | α-2 adrenoceptor antagonist | Distilled water | 5 | 45 |

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# CHAPTER III

## Noradrenaline Acts Through β-adrenergic Receptors to Enhance Induction and Maintenance of LTP

## 1. Introduction

Neuromodulatory transmitters influence cellular excitability, plasticity, and network activity in numerous brain structures, including the hippocampus (Kaczmarek and Levitan, 1987). Specifically, the hippocampus is densely innervated with noradrenergic fibres from the locus coeruleus (Moore and Bloom, 1979; Morrison and Foote, 1986). Release of noradrenaline (NA) from these fibres is implicated in hippocampal memory function (Ji et al., 2003; Brown and Silva, 2004). NA activates α1-, α2-, and β-adrenergic receptor subtypes, all of which are differentially expressed in both hippocampal principal cells and interneurons (Pieribone et al., 1994; Happe et al., 2004; Hillman et al., 2005).

Noradrenergic receptors couple to guanine nucleotide-binding regulatory proteins (G-proteins) to mediate intracellular signaling. Whereas β-adrenergic receptors interact with the Gs form of G-protein to stimulate adenylyl cyclase and subsequently increase cAMP, α2-adrenergic receptors couple to Gi G-proteins to decrease adenylyl cyclase activity and cAMP levels (Dismukes and Mulder, 1976; Minocherhomjee and Roufogalis, 1982; Ruffolo and Hieble, 1994; Raymond, 1995). α1-Adrenergic receptors are associated with Gq/G11 G-proteins, and they signal through phospholipase C (Sirvio and MacDonald, 1999). As such, the physiologic outcome of NA release depends on which receptor subtype is predominantly activated.

Activation of specific noradrenergic receptor subtypes potently modulates synaptic plasticity in the hippocampus (Katsuki et al., 1997; Sirvio and MacDonald, 1999). Synaptic plasticity is the activity-dependent modification of synaptic strength. Long-term potentiation (LTP) and long-term depression (LTD) are two types of synaptic

plasticity that likely serve as cellular mechanisms of information storage in the mammalian brain (Bliss and Collingridge, 1993; Martin and Morris, 2002). In area CA1 of the hippocampus, synaptic plasticity is highly correlated with certain forms of memory (Tsien et al., 1996; Abel et al., 1997; Gruart et al., 2006).

Application of β-adrenergic receptor agonists to area CA1 facilitates the induction and maintenance of LTP (Thomas et al., 1996; Gelinas and Nguyen, 2005). α1-Adrenergic receptor agonists have weak effects on LTP (Pussinen and Sirvio, 1998), but they can also induce LTD (Scheiderer et al., 2004). Furthermore, specific activation of α2-adrenergic receptors facilitates presynaptic inhibition of both NA and glutamate (Glu) release (Dixon et al., 1979; Boehm et al., 1999).

As such, a critical question arises: How does the endogenous neuromodulator NA affect synaptic plasticity in area CA1? Previous research suggests that NA lowers the threshold for induction of LTP (Katsuki et al., 1997). Here, I use electrophysiological methods to further investigate the effects of NA on long-term synaptic plasticity.

## 2. Materials and Methods

### a) Animals

Female C57BL/6 mice aged 8-13 weeks (Charles River, Montreal, Quebec, Canada) were sacrificed by cervical dislocation and decapitation for electrophysiology experiments. All animals were housed at the University of Alberta according to guidelines approved by the Canadian Council on Animal Care.

### b) Electrophysiology

Hippocampal slices were prepared as in Chapter II. Sliced tissue was allowed to recover for at least an hour before commencing electrophysiological measurements. Extracellular field excitatory postsynaptic potentials (fEPSPs) were obtained from area CA1 by positioning stimulating and recording electrodes in stratum radiatum. Baseline fEPSPS were evoked once per minute, at 40% of maximal fEPSP amplitude (Schimanski et al., 2002; Gelinas and Nguyen, 2005). Subsequent electrical stimulation consisted of low-frequency stimulation (LFS) at 5 Hz for 3 min.

c) Drugs

The neurotransmitter noradrenaline [NA, L(-)-noradrenaline bitartrate, 10 μM; Sigma-Aldrich Canada, Oakville, ON, Canada] was prepared daily as a concentrated stock solution at 10 mM in distilled water. The stock solution was refrigerated to prevent decomposition. The β1/β2-adrenergic receptor antagonist propranolol [(±)-propranolol hydrochloride, 50 μM; Research Biochemicals, Natick, MA] was dissolved in distilled water to create a 50 mM stock solution. Gentle heating was used to fully dissolve this compound. The α1-adrenergic receptor antagonist prazosin [prazosin hydrochloride, 10 μM; Sigma-Aldrich Canada, Oakville, ON, Canada] and the α2-adrenergic receptor antagonist yohimbine [yohimbine hydrochloride, 5 μM; Sigma-Aldrich Canada, Oakville, ON, Canada] were prepared as concentrated stock solutions at 10 mM in methanol and 5 mM in distilled water, respectively. The final concentration of methanol did not affect basal synaptic transmission (data not shown).

NA was bath applied to hippocampal slices for 15 min. All receptor antagonists were applied 20 min before NA, throughout NA application, and 10 min after NA.

Experiments were performed under dimmed light conditions due to light sensitivity of drugs. Experiments using different drugs were interleaved.

d) Data Analysis

Baseline slope values were acquired from average fEPSP slopes during a 20 min period of stimulation at once a minute. Subsequent slope values were normalized to this baseline average. Mean fEPSP slopes for statistical comparisons were measured 15 min after NA application and 120 min after 5 Hz 3 min stimulation. Statistical analysis of two groups was accomplished using Student's t-test, and analysis of three groups was performed with one-way ANOVA and Tukey-Kramer *post hoc* tests. Significance criteria for both tests was $p < 0.05$. All values are shown as mean ± standard error (SEM) with n = number of slices.

**3. Results**

a) NA acts on β-adrenergic receptors to transiently increase synaptic strength

Application of specific β-adrenergic receptor agonists in hippocampal area CA1 transiently enhances synaptic strength, whereas α-adrenergic receptor agonists can persistently decrease synaptic strength (Thomas et al., 1996; Scheiderer et al., 2004; Gelinas and Nguyen, 2005). Both α- and β-adrenergic receptors are activated by release of NA. I investigated the effects of NA on synaptic strength, and the contributions of different noradrenergic receptor subtypes to these effects.

Perfusing hippocampal slices with 10 μM NA for 15 min elicited a small but significant increase in synaptic strength compared to baseline (**Figure 3.1A:** fEPSPs were potentiated to 128.4 ± 9.4% 15 min after commencing NA application; $p < 0.05$

compared to baseline). This enhancement occurred during drug application and faded soon after drug washout. Test stimuli were applied once per minute to assess the effect of NA on fEPSP slopes. This frequency of synaptic stimulation alone does not alter basal synaptic transmission.

Concurrent blockade of α1- and α2-adrenergic receptors with the receptor antagonists prazosin and yohimbine, respectively, did not inhibit the facilitatory effect of NA on synaptic strength (**Figure 3.1B:** fEPSPs were potentiated to 129.9 ± 4.5% 15 min after commencing NA application; $p < 0.05$ compared to baseline). However, administration of the β-adrenergic receptor antagonist propranolol prior to NA application completely prevented the facilitation (**Figure 3.1C:** fEPSPs were 102.8 ± 6.1% of baseline 15 min after commencing NA application; $p > 0.5$ compared to baseline). Thus, NA can elicit a transient, β-adrenergic receptor-dependent enhancement of synaptic strength in area CA1in the absence of concurrent patterned electrical stimulation.

b) Pairing NA application with LFS generates β-adrenergic receptor-dependent LTP

Although much is known about the effects of either α- or β-adrenergic receptor activation on synaptic plasticity in area CA1, fewer studies concentrate on the interaction of these receptor subtypes during application of NA. Activation of β-adrenergic receptors potently enhances the response of CA1 pyramidal neurons to low-frequency stimulation (LFS), but is not required for long-lasting LTP induced by multiple trains of high-frequency stimulation (HFS) (Dunwiddie et al., 1982; Sarvey et al., 1989; Swanson-Park et al., 1999; Murchison et al., 2004). Conversely, activation of α-adrenergic receptors

can elicit LTD (Scheiderer et al., 2004). I therefore investigated the physiological outcome of activating noradrenergic receptors during LFS.

LFS at 5 Hz for 3 min normally induces a transient decrease in synaptic strength that quickly recovers to baseline values (Woo and Nguyen, 2002; Gelinas and Nguyen, 2005). This stimulation protocol can generate either LTP or LTD depending on the metaplastic state of synapses (Thomas et al., 1996; Liang et al., 2002; Azad et al., 2004), and it is therefore ideal for examining the bi-directional modulation of plasticity by NA. Application of NA during LFS resulted in the induction of long-lasting LTP (**Figure 3.2A:** fEPSPs were potentiated to 132.8 ± 13.8% 120 min after LFS).

Co-application of prazosin and yohimbine to block α-adrenergic receptors did not alter the LTP induced by pairing NA with LFS (**Figure 3.2B:** fEPSPs were potentiated to 140.7 ± 10.1% 120 min after LFS). Conversely, the β-adrenergic receptor antagonist propranolol blocked the induction and maintenance of this LTP (**Figure 2C:** fEPSPs were 89.9 ± 4.7% 120 min after LFS). ANOVA analysis comparing fEPSP slopes 120 min after conjoint application of LFS and NA in the presence of propranolol, prazosin and yohimibine, or no antagonist, met significance criteria ($F_{(2,13)} = 6.038$; $p < 0.05$). *Post hoc* tests revealed that the propranolol group differed from both the prazosin and yohimbine group, and from the NA-alone group ($p < 0.05$). Furthermore, the prazosin and yohimbine group did not differ significantly from the NA group ($p > 0.5$). NA can therefore act via β-adrenergic receptors to enhance the potency of LFS and facilitate both the induction and stability of LTP.

**4. Discussion**

Neurmodulators can have potent and widespread effects on synaptic plasticity. My results suggest that NA importantly influences the induction and maintenance of

plasticity in the hippocampus. Application of NA to area CA1 can either transiently or persistently enhance synaptic strength, depending on the amount of concurrent synaptic activation. NA during baseline test stimulation elicits a reversible increase in synaptic strength, whereas NA paired with LFS generates long-lasting LTP. This NA-dependent facilitation of plasticity requires β-, but not α-adrenergic receptors.

Application of β-adrenergic receptor agonists can mimic NA-dependent effects on synaptic plasticity. The β-adrenergic receptor agonist, isoproterenol, also induces a small increase in synaptic strength when applied to area CA1 (Thomas et al., 1996; Gelinas and Nguyen, 2005), and pairing this agonist with LFS at 5 Hz for 3 min elicits stable LTP (Gelinas and Nguyen, 2005). However, NA does not induce LTP as potently or reliably as isoproterenol (personal observations). Correspondingly, isoproterenol activates β1- and β2-adrenergic receptors more strongly than NA (Neve et al., 1986; Woods et al., 1989). Concurrent activation of α-adrenergic receptors during NA application could further explain the slightly different physiologic outcomes of stimulating β-adrenergic receptors with different agonists.

How does activation of β-adrenergic receptors with NA facilitate LTP induction and maintenance? Stimulation of β-adrenergic receptors increases the influx of calcium through NMDA receptors, thereby activating intracellular kinases crucial for induction of LTP (Raman et al., 1996). Furthermore, β-adrenergic receptors initiate downstream signaling via cAMP-dependent protein kinase (PKA) and extracellular-signal regulated kinase (ERK) (Thomas et al., 1996; Winder et al., 1999; Giovannini et al., 2001). These kinase cascades are crucial for the induction and stability of LTP (for review, see Nguyen and Woo, 2003; Sweatt, 2004).

Although NA also activates α-adrenergic receptors, my results indicate that they do not play a critical role in the observed forms of NA-dependent synaptic plasticity. The contribution of these receptor subtypes to plasticity may be more easily observable *in vivo*. Presynaptic α2-adrenergic receptors are the main regulators of NA release, preventing further release when bound by NA diffusing from the synaptic cleft (Dixon et al., 1979; Yavich et al., 2005). By regulating the amount of NA release, α2-adrenergic receptors could importantly affect the activation profile of postsynaptic receptors and subsequent cellular responses to NA. This regulatory function would not be evident during exogenous application of agonists *in vitro*.

The concentration of applied NA also influences postsynaptic receptor activation. *In vitro*, high concentrations of NA (>10 μM) decrease pyramidal cell excitability, presumably by preferential activation of α1-adrenergic receptors. Lower concentrations of NA increase excitability, mimicking selective β-adrenergic receptor activation (Mueller et al., 1981; Mynlieff and Dunwiddie, 1988; Rutecki, 1995). In line with these observations, application of 40 μM NA to area CA1 induces α1-adrenergic receptor-dependent LTD (Scheiderer et al., 2004). The amount and duration of NA release into the synaptic cleft could therefore bidirectionally alter synaptic strength, depending on the predominant receptor subtype activated.

The modulation of synaptic plasticity by NA may also be age-dependent. Application of NA to hippocampal slices from young rats (3-5 weeks) causes a reversible depression of fEPSP slopes (Katsuki et al., 1997; Scheiderer et al., 2004). In contrast, my data from 8-13 week old mice demonstrated a transient facilitation of synaptic strength in response to NA. LTD is robust and reliably elicited by LFS at mouse and rat hippocampal synapses only during a critical period of development. Tissue taken from mature animals (> 3 weeks for mice, > 5 weeks for rats) does not exhibit LFS-induced LTD (Dudek and Bear,

1993; Milner et al., 2004). As such, the increased propensity of young synapses to express LTD may influence the physiological response to NA.

NA facilitates hippocampus-dependent memory mainly through activation of β-adrenergic receptors (Izquierdo et al., 1998; Ji et al., 2003a; 2003b; Murchison et al., 2004). My results suggest that β-adrenergic receptors are also predominantly responsible for the effects of NA on synaptic plasticity in area CA1 of the hippocampus. Because extensive correlative evidence supports the notion that synaptic plasticity is a mechanism for memory storage (Doyere and Laroche, 1992; Abel et al., 1997; McKernan and Shinnick-Gallagher, 1997; Genoux et al., 2002; Abraham and Robins, 2005), β-adrenergic receptor-dependent increases in synaptic strength may contribute to the enhancement of memory by endogenously released NA (Cahill et al., 1994; McGaugh, 2002; Berridge and Waterhouse, 2003).

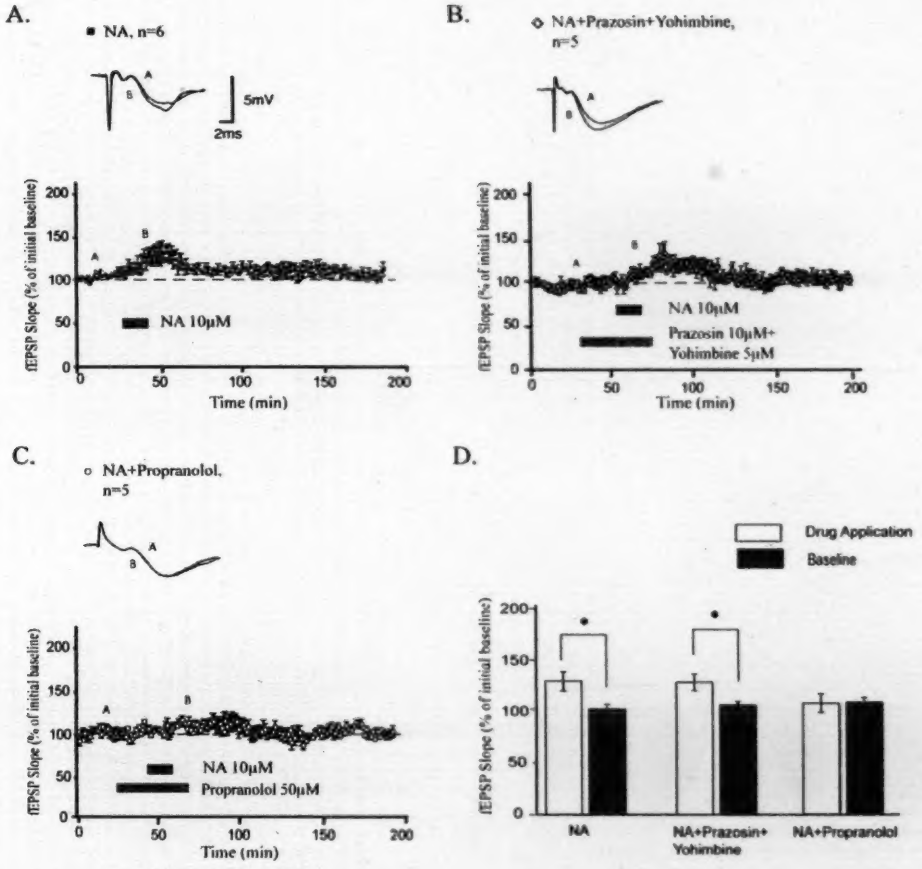


Figure 3.1: NA induces a transient, β-adrenergic receptor-dependent increase in synaptic strength. A: NA alone elicits a transient increase in fEPSP slopes. B: Application of prazosin and yohimbine does not inhibit the increase in fEPSP slopes generated by NA application. C: Application of propranolol prevents the facilitation of fEPSP slopes generated by NA application. D: Summary histogram for these experiments (*$p<0.05$). Sample traces were taken 10 min after commencement of baseline recording and 15 min after commencing NA application. Calibration: 5 mV, 2 ms.

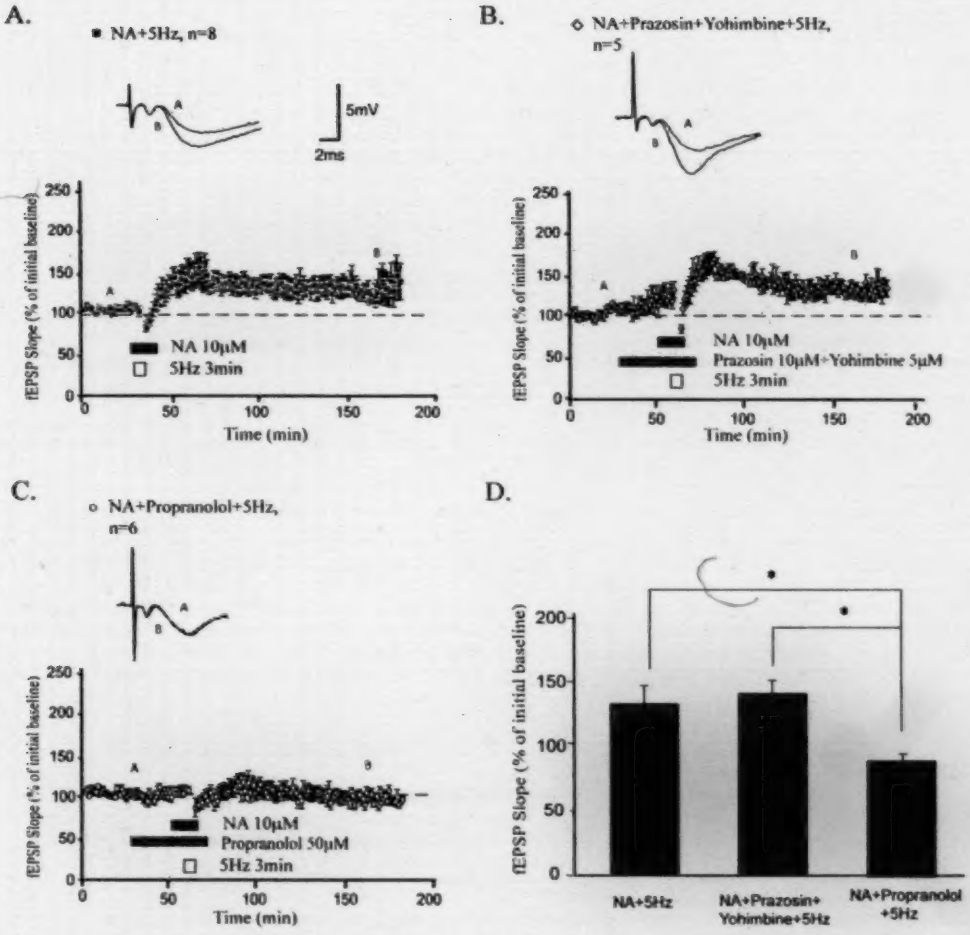


Figure 3.2: NA facilitates induction and persistence of LTP by activating β-adrenergic receptors. A: NA alone induces long-lasting LTP when paired with 5 Hz stimulation. B: Application of prazosin and yohimbine does not inhibit the induction or maintenance of LTP generated by pairing NA with 5 Hz stimulation. C: Application of propranolol inhibits induction of LTP when NA is paired with 5 Hz stimulation. D: Summary histogram for these experiments (*$p<0.05$). Sample traces were taken 10 min after commencement of baseline recording and 120 min after 5 Hz stimulation. Calibration: 5 mV, 2 ms.

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# *CHAPTER IV

## β-Adrenergic Receptor Activation Facilitates Induction of a Protein Synthesis-Dependent Late Phase of LTP

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## 1. Introduction

Enhancement of hippocampal synaptic strength ("long-term potentiation", LTP) can critically regulate storage of information in the mammalian brain (Bliss and Collingridge, 1993; Moser et al., 1998; Martin et al., 2000; Brun et al., 2001; Nathe and Frank, 2003). Like behavioural memory, LTP exhibits at least two mechanistically distinct temporal phases (Krug et al., 1984; Davis and Squire, 1984). Early phase LTP (E-LTP) does not require new protein synthesis, whereas the late phase of LTP (L-LTP), like long-term memory, requires macromolecular synthesis (Stanton and Sarvey, 1984; Davis and Squire, 1984; Deadwyler et al., 1987; reviewed in Huang et al., 1996, and Kandel, 2001). Some forms of L-LTP also require transcription (Nguyen et al., 1994). There is strong correlative evidence that L-LTP contributes to hippocampal consolidation of long-term memory (Doyere and Laroche, 1992; Bourtchouladze et al., 1994; Abel et al., 1997; Jones et al., 2001; Genoux et al., 2002).

The ability of neuromodulatory transmitters to modify synaptic plasticity thresholds constitutes a form of activity-dependent "metaplasticity" (Abraham and Bear, 1996). One neuromodulator that is prominent in the hippocampus is noradrenaline. The hippocampus receives abundant innervation from noradrenergic afferents (Loy et al., 1980). Activation of β–adrenergic receptors is involved in enhancement of memory following emotional experiences (McGaugh, 1989; Cahill et al., 1994). Hippocampal β-adrenergic receptor activation can also initiate signaling pathways known to be critical for LTP (Segal 1982; Stanton and Sarvey 1985; Madison and Nicoll, 1986). Application of β-adrenergic receptor agonists induces long-lasting changes in synaptic strength in the perforant pathway (Dahl and Sarvey, 1989) and also enhances LTP in the mossy fibre

pathway (Hopkins and Johnston, 1984, 1988; Huang and Kandel, 1996). At Schaeffer collateral/commissural CA1 synapses, β-adrenergic receptor activation alone is insufficient to elicit LTP, but it does enhance the effectiveness of subthreshold stimuli for inducing LTP (Thomas et al., 1996; Katsuki et al., 1997). Specifically, low-frequency stimulation of Schaeffer collaterals in area CA1 of mouse hippocampal slices elicits robust E-LTP when applied in the presence of isoproterenol, a β–adrenergic receptor agonist (Thomas et al., 1996). In the absence of isoproterenol, the same stimulation elicits no persistent change in basal synaptic strength (Thomas et al., 1996). Thus, activation of β–adrenergic receptors lowers the threshold for induction of E-LTP (Hopkins and Johnston, 1988; Thomas et al., 1996; Katsuki et al., 1997). Putative mechanisms regulating this induction effect include the cAMP/protein kinase A (PKA) system and the mitogen-activated protein kinase (MAPK) cascade (Raman et al., 1996; Winder et al., 1999). Specifically, it is unclear whether, and how, induction and stabilization of CA1 L-LTP are regulated by β-adrenergic receptors.

Does β-adrenergic receptor stimulation facilitate the induction and stabilization of L-LTP by subthreshold stimuli? If so, does this form of L-LTP require transcription and translation? I show here that activating β-adrenergic receptors during subthreshold electrical stimulation enhances induction and stabilization of L-LTP. This L-LTP requires protein synthesis, but not transcription. Thus, my findings show that β-adrenergic receptors control metaplasticity of L-LTP by engaging local protein synthesis.

## 2. Materials and Methods

a) Animals

Female C57BL/6 mice, 8-12 weeks of age (Charles River, Montreal, Canada) were used for all experiments. Animals were housed at the University of Alberta using guidelines approved by the Canadian Council on Animal Care.

b) Electrophysiology

Transverse hippocampal slices were prepared and maintained as described in Chapter II. For experiments on isolated CA1 dendrites, hippocampal slices were cut in ice-cold ACSF and perfused in an interface chamber for one hour. Two incisions were then made under a dissecting microscope. One cut was applied in stratum radiatum, adjacent to the cell body layer of area CA1, and another incision was created in area CA3 (Woo and Nguyen, 2003). Subsequently, slices were allowed to recover for another hour in the interface chamber before recording commenced. The following criteria were used to assess successful isolation of CA1 dendrites from CA1 pyramidal somata (Woo and Nguyen, 2003): (1) absence of a population spike when the recording electrode was placed at the cell body layer and strong stimulation was applied in stratum radiatum below the cut, and (2) absence of a fEPSP in stratum radiatum below the incision when stimulation was applied at the basilar dendrites in stratum oriens.

LTP was induced by applying one train of high-frequency stimulation (HFS; 100 Hz, 1 s duration at test strength). Depotentiation (DPT) was induced by applying low-frequency stimulation (LFS) consisting of 5 Hz for 3 min.

c) Drugs

The β-adrenergic receptor agonist, isoproterenol (ISO; R(-)-isoproterenol(+)-bitartrate, 1 μM; Sigma, St. Louis, MO) and the β-adrenergic receptor antagonist,

propranolol ( (±)-propranolol hydrochloride, 50 μM; Research Biochemicals Incorporated, Natick, MA) were prepared daily as concentrated stock solutions at 1 mM and 50 mM, respectively, in distilled water. Two different inhibitors of protein synthesis, anisomycin (25 μM; Sigma, St. Louis, MO), and emetine (20 μM; Sigma, St. Louis, MO) were prepared as concentrated stock solutions at 25 mM in DMSO and 20 mM in distilled water, respectively. Both anisomycin and emetine, at lower concentrations that those used here, blocked protein synthesis by >80% in hippocampal slices (Stanton and Sarvey, 1984; Frey et al., 1988). The stock solution (25 mM) of a transcriptional inhibitor, actinomycin D (ACT-D, 25 μM bath concentration; Bioshop Canada, Burlington, Ontario, Canada) was prepared in DMSO. At the bath concentration used here, ACT-D has been shown to block transcription by >70% in hippocampal slices (Nguyen et al., 1994). A D1/D5 antagonist, SCH 23390 (1 μM; Sigma, St. Louis, MO), was prepared as a 1 mM stock solution in water. An ERK kinase (MEK) inhibitor, PD 98059 (50 μM; Sigma, St. Louis, MO), was prepared in DMSO at a concentration of 10 mM. Each drug was diluted in ACSF to the desired final concentration and then bath applied. The final concentration of DMSO did not affect either basal synaptic transmission or LTP (data not shown). ISO was applied for a total duration of 15 min, starting 10 min prior to stimulation protocols. Propranolol and PD 98059 were applied 30 min prior to ISO application, and were present during ISO application. Anisomycin, emetine, actinomycin D, and SCH 23390 were applied 20 min prior to ISO application, and were present throughout ISO application and 10 min after ISO application. All drug experiments were performed under dimmed light conditions due to light sensitivity of drugs. Drug experiments were interleaved with drug-free controls.

d) Data Analysis

The initial slope of the fEPSP was measured as an index of synaptic strength (Johnston and Wu, 1995). Average 'baseline' slope values were acquired over a period of 20 min before experimental protocols were applied. fEPSP slopes were measured at 120 min after LFS or HFS for comparisons of late LTP. Student's t-test was used for statistical comparisons of mean fEPSP slopes between two groups, with a significance level of $p < 0.05$. One-way ANOVA and Tukey-Kramer *post hoc* tests were done for comparison of more than two groups to determine which groups were significantly different from the others. The Welch correction was applied in cases in which the standard deviations of groups being compared were significantly different. All values shown are means ± standard errors (SEM), with n = number of slices.

**3. Results**

a) β-Adrenergic receptor activation facilitates expression of a late phase of LTP

Activation of β-adrenergic receptors in the CA1 region of mouse hippocampal slices has been shown to enhance induction of LTP by stimulation protocols that normally have no persistent effect on fEPSPs (Thomas et al., 1996; for rat data in CA3 and CA1, see also Hopkins and Johnston, 1988; Katsuki et al., 1997). I tested the idea that β-adrenergic receptor activation enhances expression of L-LTP. Application of a β-adrenergic receptor agonist, isoproterenol (ISO, 1 μM), to hippocampal slices for 15 min induced a small enhancement of synaptic transmission in area CA1 that faded soon after drug washout (**Figure 4.1A:** fEPSPs were 105.3 ± 4.2% of baseline slopes, 60 min after ISO application). Similarly, 5 Hz stimulation for 3 min to the Schaeffer collateral-

commissural fibers in CA1 elicited only a transient depression of synaptic transmission that quickly recovered to baseline levels (**Figure 4.1B:** fEPSPs were 101.4 ± 3.5% of baseline, 60 min after 5 Hz stimulation). However, 5 Hz stimulation delivered in the presence of ISO induced LTP that persisted for over 2 hours (**Figure 4.1B:** fEPSPs were potentiated to 151.5 ± 10.9% 120 min after 5 Hz stimulation, $p < 0.01$ compared to 5 Hz alone). Because E-LTP can decay within 2 hours in these conditions (Huang and Kandel, 1994), these results suggest that pairing β-adrenergic receptor activation with 5 Hz LFS elicits stable L-LTP. To test the idea that facilitation of L-LTP expression by ISO depends on the induction protocol, I examined LTP generated by one train of 100 Hz stimulation (1 s duration). This procedure induces E-LTP in mouse hippocampal slices (Duffy et al., 2001) (**Figure 4.1C:** fEPSPs were 102.3 ± 5.5% 120 min after 100 Hz). When ISO was applied during the single train, the persistence of LTP was enhanced (**Figure 4.1C:** fEPSPs were potentiated to 155.4 ± 14.1% 120 min after 100 Hz, $p < 0.01$ compared to 1 x 100 Hz alone). Thus, β-adrenergic receptor activation enhances the ability of stimuli to induce a late phase of LTP. The combined data from both 5 Hz and 1 x 100 Hz stimulation underscore the idea that the enhancement of persistence of LTP by β-adrenergic receptor activation is not restricted to particular stimulation protocols.

b) LTP elicited by isoproterenol application during subthreshold stimulation requires β-adrenergic receptors and MAP kinase, but not dopamine receptors

Induction of LTP by low frequency electrical stimulation paired with ISO application was inhibited by a β-adrenergic antagonist, propranolol (**Figure 4.2A:** fEPSPs were 106.5 ± 6.8% of baseline 60 min after 5 Hz stimulation). This LTP therefore requires activation of β-adrenergic receptors. However, activation of dopamine

D1/D5 receptors has also been shown to increase expression of early and late LTP in area CA1 (Huang and Kandel, 1995; Otmakhova and Lisman, 1996). To ascertain that my observed enhancement of LTP by ISO was not also mediated by dopamine receptors, I applied a D1/D5 receptor antagonist, SCH 23390. Induction and maintenance of LTP elicited by pairing ISO with 5 Hz stimulation was not inhibited in the presence of SCH 23390 (**Figure 4.2B:** fEPSPs were potentiated to 158.8 ± 11.6% 120 min after 5 Hz stimulation, $p > 0.5$ compared to ISO+5 Hz alone). These data show that ISO selectively activates β-adrenergic receptors to establish LTP.

Induction of LTP in the presence of ISO and 5 Hz stimulation is dependent on the intracellular cAMP cascade (Raman et al., 1996; Winder et al., 1999). However, the signaling pathways involved in the facilitated maintenance of LTP by β-adrenergic receptor activation are unknown. As a preliminary step towards identifying these pathways, I examined the effects of a MEK inhibitor, PD 98059, on my β-adrenergic receptor-mediated L-LTP. Pairing 1x100 Hz electrical stimulation with ISO application in the presence of PD 98059 inhibited L-LTP (**Figure 4.2C:** fEPSPs were 108.9 ± 6.6% 120 min after 1x100 Hz, $p < 0.01$ compared to ISO+1x100 Hz alone). Because LTP induced by 1x100 Hz stimulation is independent of the MAP kinase cascade (Winder et al., 1999), my data suggest that β-adrenergic receptor activation recruits MAP kinases to stabilize LTP.

c) β-Adrenergic receptor activation paired with subthreshold stimulation renders L-LTP immune to depotentiation

Depotentiation (DPT) is activity-induced reversal of LTP that can occur during a restricted time interval immediately after LTP induction (Barrionuevo et al., 1980;

Staubli and Lynch, 1990; Fujii et al., 1991; Bashir and Collingridge, 1994; Huang et al., 1999). Low frequency stimulation (LFS), such as 5 Hz for 3 min, can elicit depotentiation (O'Dell and Kandel, 1994; see also Staubli and Lynch, 1990, and Fujii et al., 1991). Furthermore, susceptibility to DPT has been shown to depend on molecular mechanisms associated with the maintenance of L-LTP (Barco et al., 2002; Woo and Nguyen, 2002; 2003). Processes involved in the cellular consolidation of E-LTP to L-LTP can also confer immunity to depotentiation (Woo and Nguyen, 2003).

Is L-LTP generated by pairing β-adrenergic receptor activation with LFS also immune to DPT? To address this question, I paired ISO application with 5 Hz LFS to induce L-LTP, allowed 10 min for ISO washout, and then administered LFS (5 Hz 3 min) in an attempt to depotentiate the L-LTP. I found that L-LTP could not be persistently erased; fEPSPs recovered to pre-DPT, potentiated values (**Figure 4.3A:** fEPSPs were 140.8 ± 11.2% and 143.9 ± 9.0%, pre-DPT and 120 min post-DPT respectively, $p > 0.5$).

Next, I examined whether LTP induced by ISO application paired with one train of HFS was similarly immune to DPT. LFS applied 10 min after one train of HFS alone caused an immediate and persistent reversal of LTP (**Figure 4.3B:** fEPSPs were 133.0 ± 8.0% and 101.0 ± 4.4%, pre-DPT and 120 min post-DPT respectively, $p < 0.01$). However, application of ISO during one train of HFS alters the properties of the induced LTP. In this case, LTP did not persistently depotentiate, and it recovered to pre-DPT values (**Figure 4.3B:** fEPSPs were 158.1 ± 12.6% and 155.5 ± 10.4% pre-DPT and 120 min post-DPT respectively, $p > 0.5$).

Because pairing ISO with LFS induces LTP, it was important to ensure that the ISO had been washed out prior to the LFS given for DPT. To test this assumption, I

administered ISO for 15 min, allowed 10 min for drug wash-out, then applied 5 Hz LFS to mimic the DPT stimulus. I found that in this situation, the LFS did not induce LTP (**Figure 4.3C:** fEPSPs were 104.7 ± 5.8% 30 min after LFS). Therefore, a 10 min interval is sufficient to allow for drug washout, and depotentiation experiments were not confounded by interaction of the 5 Hz DPT stimulus with ISO. Activation of β-adrenergic receptors thus triggers pathways that not only facilitates induction and expression of L-LTP, but makes this L-LTP immune to persistent reversal by depotentiating stimuli.

d) Protein synthesis is required for L-LTP induced by β-adrenergic receptor activation paired with subthreshold stimulation

*De novo* protein synthesis is a hallmark of L-LTP. Whereas E-LTP is protein synthesis-independent, L-LTP requires protein synthesis for its maintenance (Stanton and Sarvey, 1984; Deadwyler et al., 1987; Frey et al., 1988; Nguyen and Kandel, 1996; reviewed in Huang et al., 1996 and Kandel, 2001). Furthermore, studies have shown that protein synthesis is required for synaptic immunity to DPT (Woo and Nguyen, 2003). I therefore investigated whether L-LTP generated by activating β-adrenergic receptors during 5 Hz LFS or 1 x 100 Hz stimulation resembles previously characterized forms of L-LTP by requiring protein synthesis.

Firstly, anisomycin, an inhibitor of translation, was bath applied at a concentration that inhibits more than 80% of protein synthesis (Frey et al., 1988). L-LTP elicited by pairing β-adrenergic receptor activation with 5 Hz LFS decayed in the presence of anisomycin (**Figure 4.4A:** fEPSPs were 115.1% ± 10.3% 120 min after LFS). To exclude the possibility that the decay in LTP could be attributed to effects of anisomycin

unrelated to protein synthesis, I repeated this experiment using another translation inhibitor, emetine. Bath application of emetine likewise inhibited L-LTP when ISO was administered during 5-Hz LFS (**Figure 4.4B:** fEPSPs were 107.6 ± 8.9% 120 min after LFS). An ANOVA that compared fEPSPs 120 min after conjoint ISO application and LFS in the presence of anisomycin, emetine, or no drug demonstrated significant differences between groups ($F_{(2, 30)} = 4.695$, $p < 0.02$). Subsequent Tukey-Kramer *post hoc* tests revealed that both anisomycin and emetine significantly inhibited L-LTP ($p < 0.05$). Furthermore, the anisomycin and emetine groups did not significantly differ from each other in their impairment of L-LTP ($p > 0.05$).

L-LTP induced by pairing β-adrenergic receptor activation with one train of HFS also required protein synthesis. Bath application of emetine caused L-LTP to gradually decay (**Figure 4.4C:** fEPSPs were 107.6 ± 2.6% 120 min after 100 Hz, $p < 0.01$ compared to ISO+1x100 Hz alone). Therefore, like L-LTP generated by multiple trains of HFS, protein synthesis is required for L-LTP induced by the activation of β-adrenergic receptors during stimulation that normally does not induce protein synthesis-dependent L-LTP. Previous studies have shown that the protein synthesis inhibitors used do not affect hippocampal slice viability (Stanton and Sarvey, 1984; Frey et al., 1988). My second pathway data also show that inhibition of protein synthesis does not affect basal synaptic transmission in hippocampal slices, consistent with these previous reports (Krug et al., 1984; Frey et al., 1988; Nguyen et al., 1994; Scharf et al., 2002).

e) Somatic transcription is not required for L-LTP induced by β-adrenergic receptor activation paired with subthreshold stimulation

Studies of long-term synaptic plasticity have focused on the roles of gene expression at the transcriptional level (Sossin, 1996; Martin and Kosik, 2002; Deisseroth et al., 2003). Transcription is needed for expression of some forms of L-LTP (Abraham et al., 1993; Nguyen et al., 1994; Frey et al., 1996; Nayak et al., 1998; Jones et al., 2001; Ying et al., 2002). I therefore investigated whether L-LTP initiated by pairing β-adrenergic receptor activation with LFS requires transcription. I used a transcription inhibitor, actinomycin D (ACT-D), at a concentration (25 μM) that has been shown to block transcription by >70% in hippocampal slices (Nguyen et al., 1994). Bath application of ACT-D did not inhibit L-LTP induced by ISO paired with LFS (**Figure 4.5A:** fEPSPs were 157.7 ± 12.1% at 120 min after LFS), indicating that transcription is not necessary for this form of L-LTP. L-LTP produced by pairing β-adrenergic receptor activation with one train of HFS is also independent of transcription. Application of ACT-D did not inhibit L-LTP induced by this stimulation protocol (**Figure 4.5B:** fEPSPs were 151.9 ± 12.0% 120 min after 100 Hz, $p > 0.05$ compared to ISO+100 Hz alone).

I hypothesized that dendritic protein synthesis may be critical for L-LTP generated in this manner. To test this idea, I recorded from slices containing isolated CA1 pyramidal cell dendrites that were created by applying two small cuts in stratum radiatum, adjacent to the cell body layers of areas CA1 and CA3 (Woo and Nguyen, 2003). Slices prepared in this manner allowed fEPSPs to be recorded from isolated, "de-somatized" CA1 dendrites (Frey et al., 1989; Woo and Nguyen, 2003). Although these slices exhibited smaller maximal fEPSP amplitudes (ranging from 1-3 mV) than intact slices, they were still capable of robust potentiation. When ISO was bath applied to these cut slices and paired with 5 Hz LFS, long-lasting potentiation was observed (**Figure**

**4.5C:** fEPSPs were 141.6 ± 10.5% 120 min after 5 Hz stimulation). Therefore, intact communication from the cell body layer to the dendrites is not required for expression of L-LTP generated by pairing β-adrenergic receptor activation with LFS.

However, it is possible that cutting hippocampal slices may alter the cellular state such that ISO application increases synaptic strength in a protein synthesis-independent manner. To exclude this possibility, I applied the protein synthesis inhibitor emetine during ISO+5 Hz stimulation in cut slices. As observed in uncut slices, emetine inhibited the L-LTP in cut slices (**Figure 4.5D:** fEPSPs were 105.7 ± 7.6% 120 min after 5 Hz stimulation). Therefore, LTP observed in the cut slice preparation is similarly dependent on protein synthesis.

ANOVA analysis followed by Tukey-Kramer *post hoc* analyses demonstrated no significant differences between results obtained from intact slices treated with ACT-D, cut slices with ISO and 5 Hz LFS, and intact slices with ISO and 5 Hz. However, these slice treatments all were significantly different from cut slices treated with ISO, emetine and 5 Hz. (**Figure 4.5E:** $F_{(3, 26)} = 5.432$, $p < 0.01$). When considered alongside the results of **Figure 4.4**, these data suggest that local, dendritic translation is needed for expression of L-LTP by conjoint activation of β–adrenergic receptors and LFS.

f) L-LTP induced by β-adrenergic receptor activation paired with subthreshold stimulation does not exhibit a late transcriptional component

In some slice preparations, L-LTP has been shown to last up to 8 hours (Frey et al., 1996). This L-LTP demonstrates a dependence on transcription that is not evident until approximately 5 hours after tetanus (Frey et al., 1996). In light of this data, I investigated whether my form of L-LTP is similarly long-lasting and dependent on

transcription at extended time periods after induction. I observed that conjoint application of ISO and 5 Hz stimulation produced non-decremental LTP that was maintained for at least 6 hours (**Figure 4.6A:** fEPSPs were 149.9 ± 16.4% 360 min after 5 Hz). The duration of this form of L-LTP is therefore comparable to the duration of HFS-induced L-LTP observed in hippocampal slices. Application of ACT-D had no effect on the maintenance of L-LTP at this time point (**Figure 4.6B:** fEPSPs were 152.9 ± 13.3% 360 min after 5 Hz). **Figure 4.6C** compares the potentiation observed between slices treated with and without ACT-D in the presence of ISO+5 Hz at various times after induction. These data show that activation of β-adrenergic receptors during LFS produces L-LTP which is stable in the absence of substantial transcription for at least 6 hours.

Overall, my results indicate that pairing β-adrenergic receptor activation with subthreshold electrical stimulation is sufficient to induce long-lasting LTP. This form of LTP can be classified as "late-phase" because of its requirement for protein synthesis, its immunity to depotentiation, and its non-decremental maintenance for at least 6 hours. However, this L-LTP differs from HFS-induced L-LTP because it is independent of somatic transcription and instead requires local, dendritic protein synthesis. My results show further that the properties of this L-LTP are generated by β-adrenergic receptor activation and depend on the downstream initiation of the MAP kinase cascade.

**4. Discussion**

Neuromodulatory transmitters play critical roles in regulating activity-dependent synaptic plasticity (Moody et al., 1999; Mann and Greenfield, 2003; van Dam et al.,

2004). Neuromodulators such as noradrenaline can alter the sensitivity of LTP to electrical activity. Such biochemically-mediated "metaplasticity" (cf. Abraham and Tate, 1997) can shift the plasticity induction profiles of synapses. My results reveal that pharmacological activation of β-adrenergic receptors enhances maintenance of LTP induced by weak electrical stimulation. This facilitation appears to require dendritic protein synthesis. Activation of noradrenergic receptors may therefore enable hippocampal neurons to respond quickly and persistently to subthreshold synaptic stimulation (Thomas et al., 1996).

Conjoint activation of β-adrenergic receptors with subthreshold electrical stimulation produces LTP that shares many properties with tetanus-induced L-LTP. This L-LTP is resistant to activity-induced reversal by depotentiating stimuli (Barco et al., 2002; Woo and Nguyen, 2002). I found that pairing β-adrenergic receptor activation with LFS induced LTP that was similarly immune to depotentiation. Because a critical step toward synaptic immunity to DPT is translation of mRNA (Woo and Nguyen, 2003), my results suggest that β-adrenergic receptor activation may engage intracellular pathways that regulate protein synthesis. Consistent with this hypothesis, anisomycin and emetine inhibited maintenance of β-adrenergic receptor-induced LTP.

Furthermore, transcription is not required for L-LTP induced by pairing β-adrenergic receptor activation with LFS. Intact maintenance of LTP in slices containing isolated CA1 pyramidal cell dendrites reinforces the idea that the soma is not necessary for this form of L-LTP. Dendritic translation appears to be needed to stabilize this L-LTP, because emetine selectively inhibited long-term stability of L-LTP in slices containing isolated dendrites. Multiple trains of HFS delivered to isolated dendrites in

mouse hippocampal slices elicit LTP that decays shortly after tetanus (Woo and Nguyen, 2003). This decay might be caused by the inability of transcriptionally-generated protein products to reach the synapse. My results suggest that some forms of LTP may initiate maintenance and counteract decay processes using only dendritic translation products.

L-LTP, like long-term memory, is characterized mainly by its extended duration and dependence on protein synthesis (Davis and Squire, 1984; McGaugh, 2000). β-Adrenergic receptor activation induces expression of these properties in response to electrical stimulation protocols that normally are unable to elicit long-term changes in synaptic strength. Stimulation of β-adrenergic receptors therefore appears to initiate a form of L-LTP. Tetanus-induced L-LTP also requires transcription (Nguyen et al., 1994), whereas my form of LTP is independent of transcription, even at extended time points. However, recent lines of research demonstrate that translational regulation may make important contributions to stability of long-lasting changes in synaptic efficacy (Kelleher et al., 2004b). For example, activation of metabotropic glutamate receptors in the hippocampus induces L-LTD that is insensitive to transcriptional inhibition (Huber et al., 2000). In agreement with my findings, this form of L-LTD reinforces the notion that translation of pre-existing mRNAs can be sufficient to maintain a late phase of synaptic plasticity.

Evidence for cellular machinery to support a specific role of dendritic translation in long-term synaptic plasticity has been found. Indeed, polyribosome complexes located in dendrites (Steward and Levy, 1982; Steward, 1983) mediate local protein synthesis in hippocampal dendrites (Steward et al., 1996). Although many of the mRNAs translated in dendrites remain uncharacterized, those that have been identified exhibit a broad range

of functions, suggesting that local dendritic translation can regulate many aspects of synaptic physiology and plasticity (Steward, 1997; Bagni et al., 2000). The presence of separate somatic and dendritic pathways by which a synapse may acquire proteins crucial for L-LTP poses two intriguing questions: How do these pathways respond to different patterns of synaptic activation? How do they interact with each other?

L-LTP induced by HFS requires transcription (Abraham et al., 1993; Nguyen et al., 1994; Frey et al., 1996; Nayak et al., 1998; Jones et al., 2001; Ying et al., 2002; reviewed in Steward and Schuman, 2001). Interestingly, various studies suggest a role for local protein synthesis in LTP that requires activation of neuromodulatory receptors rather than strong electrical stimulation. Application of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) to hippocampal slices produces L-LTP that requires dendritic protein synthesis (Kang and Schuman, 1996). Also, whereas application of the muscarinic agonist, carbachol, or electrical stimulation alone does not engage *de novo* protein synthesis, the combination of both elicits dendritic translation (Feig and Lipton, 1993). These observations parallel my results observed when β-adrenergic receptor activation is combined with LFS. Altogether, my data suggest that L-LTP mediated by β-adrenergic receptor activation requires local protein synthesis, but not somatic transcription. Multiple forms of long-lasting synaptic plasticity exist, and my findings add to a growing body of evidence that certain neuromodulators can induce a late phase of plasticity that bypasses transcriptional processes.

Plasticity processes that require translation but not transcription also exist in many invertebrate systems. Intermediate term facilitation (ITF) in Aplysia requires new protein, but not mRNA, synthesis (Ghirardi et al., 1995; Mauelshagen et al., 1996).

Similar intermediary phenomena have been observed in crayfish and Lymnaea (Beaumont et al., 2001; Sangha et al., 2003). L-LTP induced by pairing β-adrenergic receptor activation with subthreshold stimulation resembles ITF in its selective requirement of protein synthesis. Activation of neuromodulatory afferents may therefore be a translation-dependent mechanism to selectively recruit pathways that rapidly engage long-lasting, rather than transient, changes in synaptic efficacy.

*In vivo*, different neuromodulators can act together to mediate cellular events. Noradrenaline and dopamine both initiate protein synthesis-dependent L-LTP in the hippocampus when paired with subthreshold stimulation (Huang and Kandel, 1995), although these effects may depend on concentrations of the applied agonist and stimulation protocols utilized (Swanson-Park et al., 1999). However, my data with the β-adrenergic antagonist propranolol and D1/D5 dopamine antagonist SCH 23390 indicate that application of ISO induces L-LTP that is selectively dependent on β-adrenergic receptor activation. Genetic knockout studies conducted by Winder et al. (1999) further suggest that this effect is mediated by β1-adrenergic receptors only. These results raise the question of how noradrenaline and dopamine, which have similar effects on LTP, are differentially regulated.

Dopamine is required for L-LTP in area CA1 and has been linked to downstream CREB activation (Huang and Kandel, 1995; Otmakhova and Lisman, 1996; Pittenger et al., 2002). During behaviour, activation of dopaminergic receptors may play a permissive role in initiating transcriptional pathways linked to L-LTP and memory. Noradrenaline is not necessary for L-LTP in CA1 (Stanton and Sarvey, 1985), and my results suggest that some of its outcomes are regulated by dendritic translation. It is

possible that emotionally charged stimuli that trigger the noradrenergic system could engage translational mechanisms to enhance association and retention of stimuli that are initially subthreshold for activation of dopamine receptors.

How does β-adrenergic receptor activation link to protein synthesis and LTP maintenance? β-Adrenergic receptors have been shown to stimulate PKA via the cAMP pathway. PKA is also critical for L-LTP, and its downstream targets include transcription factors (Madison and Nicoll, 1986; Dunwiddie et al., 1992; Thomas et al., 1996; Brown et al., 2000, Abel et al., 1997). However, β-adrenergic receptors also activate the MAPK cascade (Winder et al., 1999; Giovannini et al., 2001). My data indicate that this cascade may be selectively critical for the late phase of some forms of β-adrenergic LTP. Furthermore, recent evidence implicates MAP kinases in translational control of synaptic plasticity (Kelleher et al., 2004a). This pathway may be important for signal transduction from the β-adrenergic receptor to protein synthesis and LTP maintenance. Further mechanistic research is required to investigate this connection.

Is enhancement of L-LTP by β-adrenergic receptor activation relevant to long-term memory? Both L-LTP and long-term memory require new protein synthesis (Davis and Squire, 1984; McGaugh, 2000). Interestingly, the β-adrenergic neuromodulatory system plays a crucial role in enhancement of memory, especially during periods of heightened emotional arousal (McGaugh, 1989; Cahill et al., 1994). Recent evidence suggests that noradrenaline may be particularly important in the complex events underlying memory retrieval (Murchison et al., 2004). Irrespective of specific

contributions to acquisition, consolidation or retrieval, it is clear that β-adrenergic receptor activation can importantly regulate memory processing.

Noradrenaline exerts its effects at several sites, including the amygdala and forebrain (Moore and Bloom, 1979). However, the importance of hippocampal area CA1 for rapid encoding of events that comprise "episodic memory" (Eichenbaum, 2000) indicates that hippocampal β-adrenergic receptor activation is crucial for initiating long-term memory storage and/or retrieval. Although the conclusion that L-LTP in general is a cellular mechanism for long-term memory storage remains debatable, there is strong correlative evidence to support this idea (Doyere and Laroche, 1992; Bourtchouladze et al., 1994; Abel et al., 1997; Jones et al., 2001; Genoux et al., 2002). My results lead me to speculate that enhancement of L-LTP by pairing β-adrenergic receptor activation with LFS may contribute to noradrenergic enhancement of memory observed in previous studies (McGaugh, 1989; Cahill et al., 1994). Such enhancement of long-term memory might be initiated by synthesis of synaptically-localized proteins, a process implicated in stabilization and enhancement of L-LTP (Barco et al., 2002, Kelleher et al., 2004a,b).

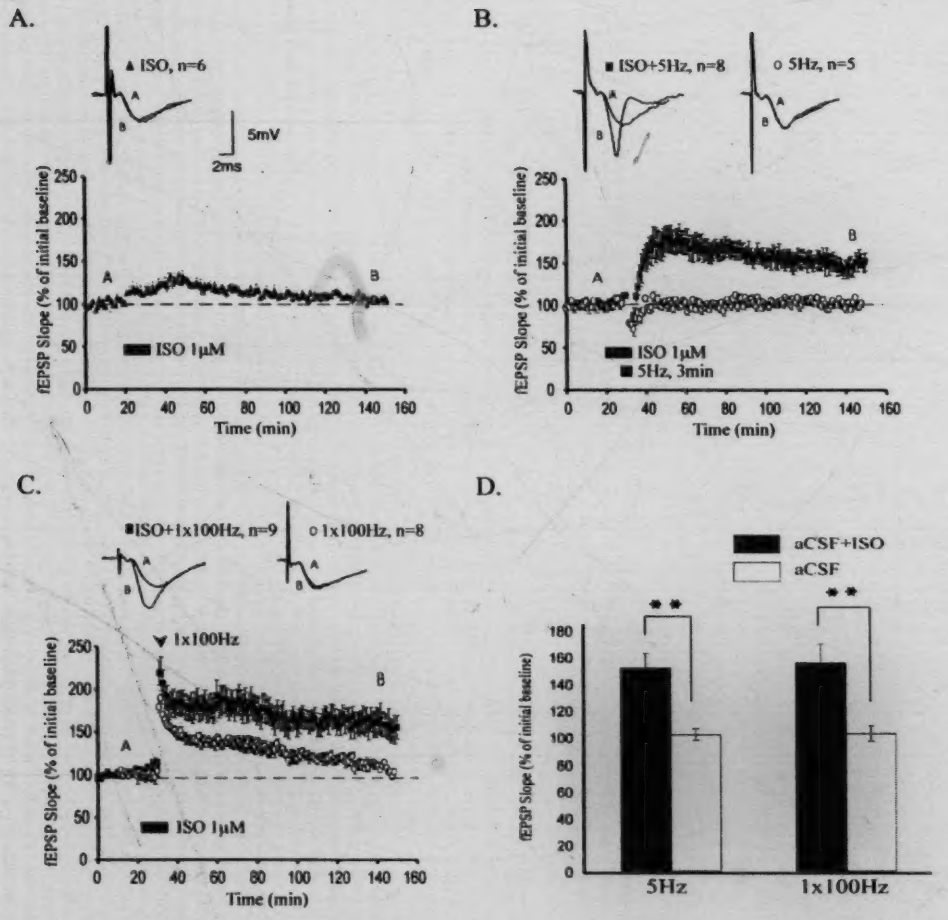


Figure 4.1: β-Adrenergic receptor activation facilitates induction and enhances persistence of L-LTP. (A) ISO application alone has no long-lasting effects on synaptic strength (filled triangles). (B) Pairing 5 Hz stimulation (for 3 min) with ISO application induces L-LTP (filled squares) whereas 5 Hz stimulation alone induces only a transient decrease in synaptic strength (open circles). (C) Pairing one train of 100 Hz stimulation with ISO application induces L-LTP (filled squares) whereas 100 Hz stimulation alone induces only E-LTP (open circles). (D) Summary histogram for these experiments (** denotes $p<0.01$). All sample traces were taken 10 min after commencement of baseline recording and 120 min after stimulation protocol. Calibration: 5 mV, 2 ms.

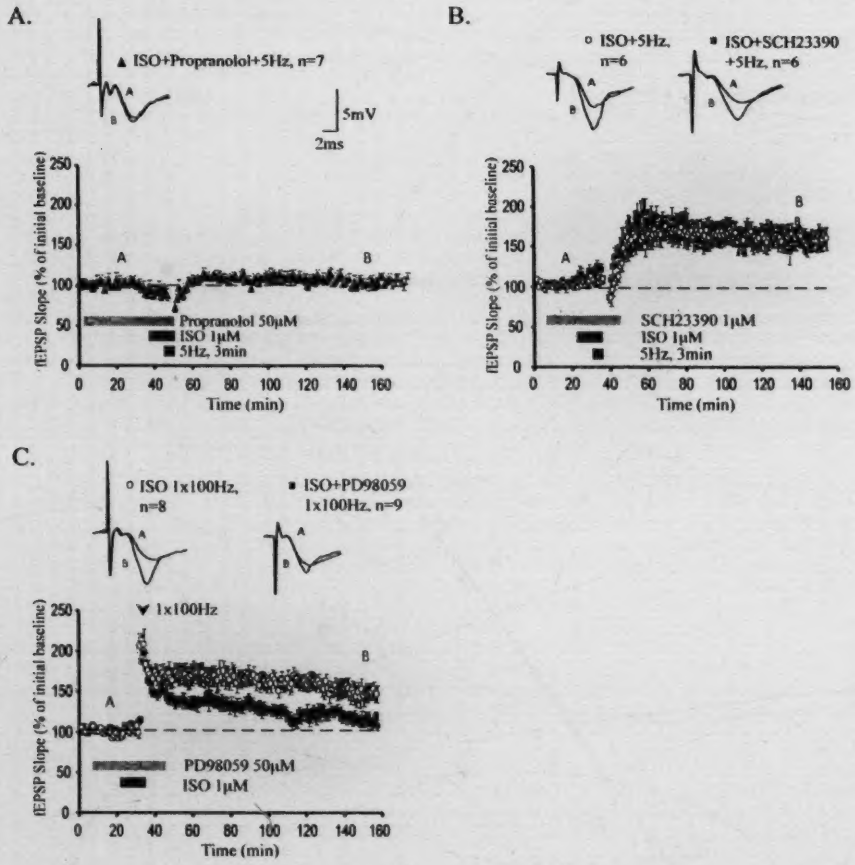


Figure 4.2: LTP elicited by ISO application during subthreshold stimulation requires β-adrenergic receptors and MAP kinase, but not dopamine receptors. (A) Application of propranolol inhibits LTP generated by pairing 5 Hz stimulation with ISO application (filled triangles). (B) Application of SCH 23390 does not inhibit LTP generated by pairing 5 Hz stimulation with ISO application (filled squares). (C) Application of PD 98059 inhibits the maintenance of LTP induced by pairing 1x100 Hz stimulation with ISO application (filled squares). All sample traces were taken 10 min after commencement of baseline recording and 120 min after stimulation protocol. Calibration: 5 mV, 2 ms.

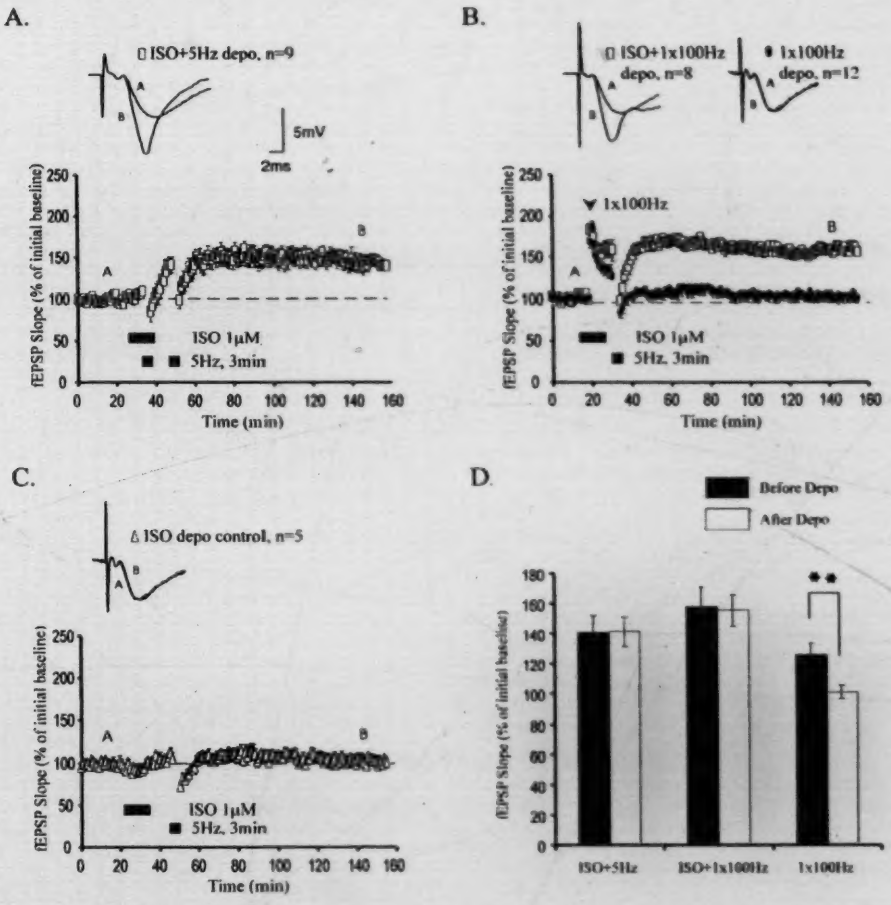


Figure 4.3: β-Adrenergic receptor activation paired with subthreshold stimulation renders LTP immune to depotentiation. (A) LFS given 10 min after 5 Hz stimulation in the presence of ISO did not persistently erase LTP (open squares). (B) LFS given 10 min after 1x100 Hz stimulation in the presence of ISO did not persistently erase LTP (open squares). In contrast, when LFS was applied 10 min after 1x100 Hz stimulation alone, persistent depotentiation was observed (filled circles). (C) LFS given after ISO application and washout did not persistently alter synaptic strength (open triangles). (D) Summary histogram for these experiments comparing fEPSP slopes measured immediately before depotentiating LFS to fEPSP slopes obtained 120 min after depotentiating LFS (** denotes $p<0.01$). All sample traces were taken 10 min after commencement of baseline recording and 120 min after depotentiating LFS stimulation. Calibration: 5 mV, 2 ms.

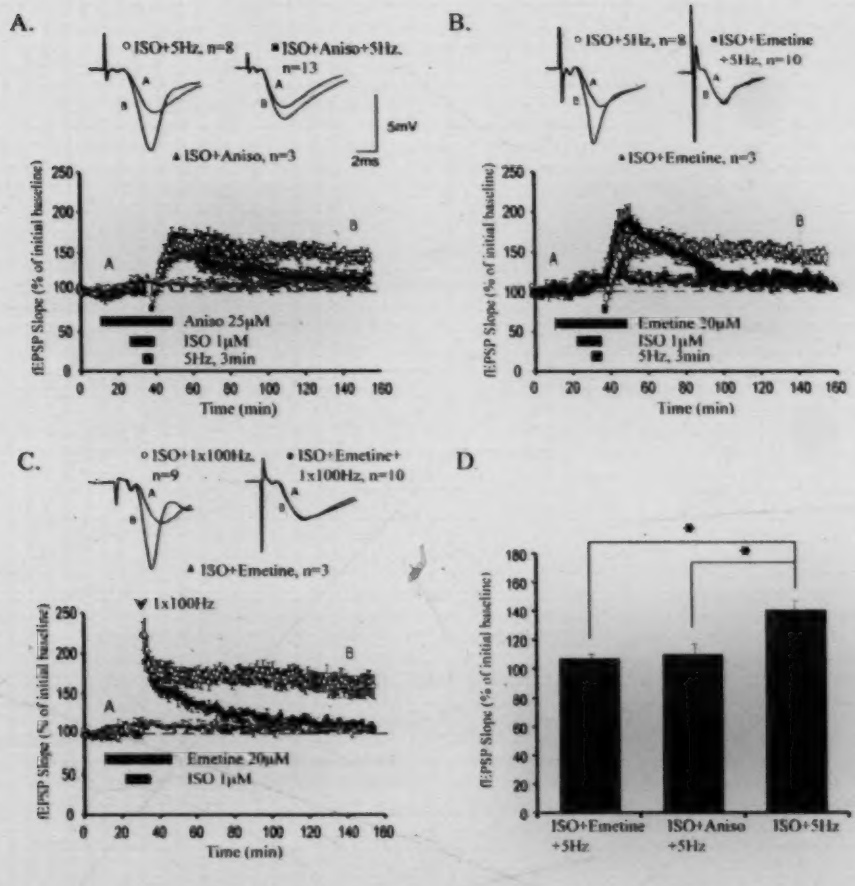


Figure 4.4: L-LTP induced by β-adrenergic receptor activation paired with subthreshold stimulation requires protein synthesis. (A) Application of anisomycin caused L-LTP generated by pairing 5 Hz LFS with ISO to decay to levels significantly below drug-free controls (filled squares). Addition of anisomycin did not alter basal synaptic transmission in a second independent pathway that did not receive 5 Hz LFS (filled triangles). (B) Application of emetine also caused L-LTP ge nerated by pairing 5 Hz LFS with ISO to decay (filled squares). Addition of emetine did not alter basal synaptic transmission in a second independent pathway that did not receive 5 Hz LFS (filled triangles). Note that transmission in these second pathways was increased in the presence of anisomycin or emetine and ISO; this increase was due to ISO (see Fig. 1A). (C) Application of emetine caused L-LTP generated by pairing 1x100 Hz stimulation with ISO to decay (filled squares). Addition of emetine again did not significantly alter basal synaptic transmission above the transient increase in synaptic strength caused by ISO application (filled triangles). (D) Summary histogram comparing effects of anisomycin and emetine on 5 Hz LFS paired with ISO application (* denotes $p<0.05$). All sample traces were taken 10 min after commencement of baseline recording and 120 min after stimulation protocol. Calibration: 5 mV, 2 ms.

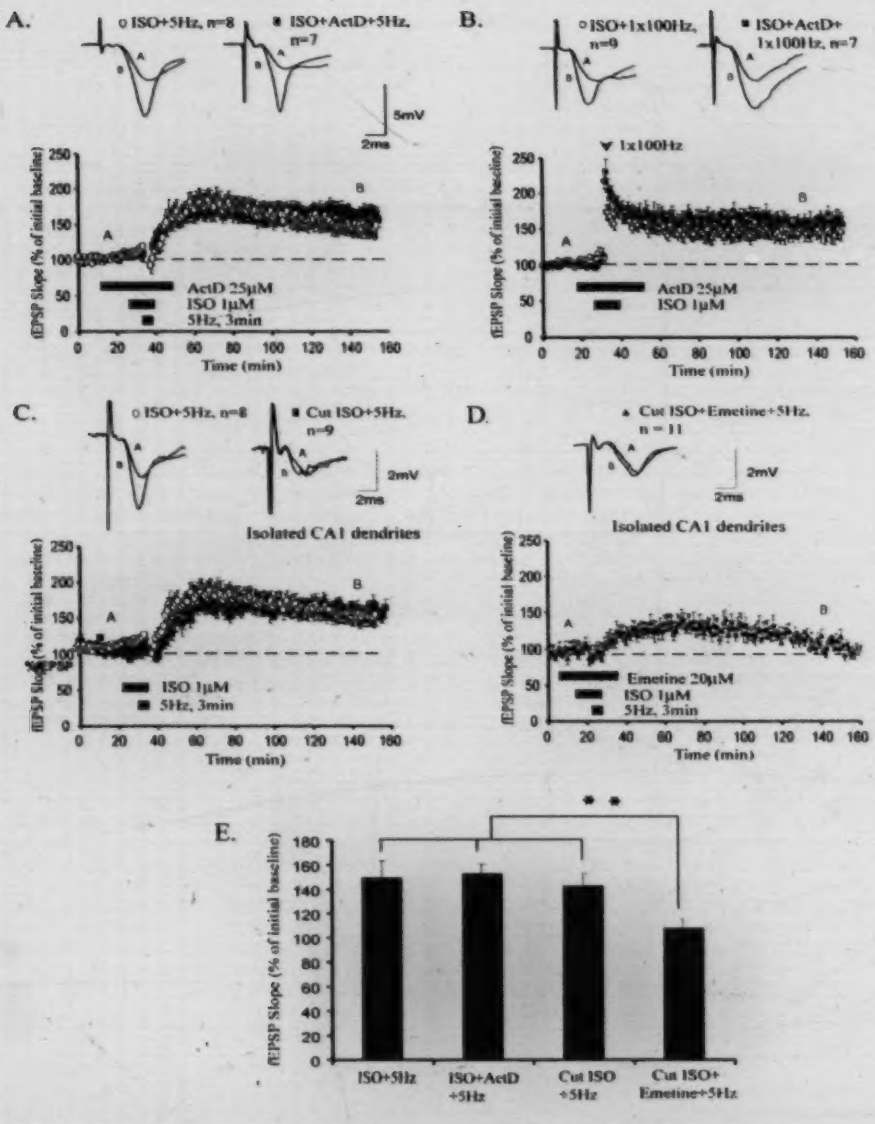


Figure 4.5: L-LTP induced by β-adrenergic receptor activation paired with subthreshold stimulation is independent of transcription. (A) Application of actinomycin D did not inhibit L-LTP e licited by pairing 5 Hz with ISO (filled squares). (B) Application of actinomycin D did not inhibit L-LTP e licited by pairing 1x100 Hz stimulation with ISO (filled squares). (C) Application of ISO during 5 Hz generated L-LTP in isolated CA1 pyramidal cell dendrites (filled squares). (D) Application of emetine to cut slices treated with ISO and 5 Hz inhibited the maintenance of L-LTP (filled triangles). (E) Summary histogram comparing effects of applying actinomycin D and emetine to isolated CA1 pyramidal dendrites (cut slices), (** denotes $p<0.01$). All sample traces were taken 10 min after commencement of baseline recording and 120 min after stimulation protocol. Calibration for cut slices: 2 mV, 2 ms. Calibration for intact slices: 5 mV, 2 ms.

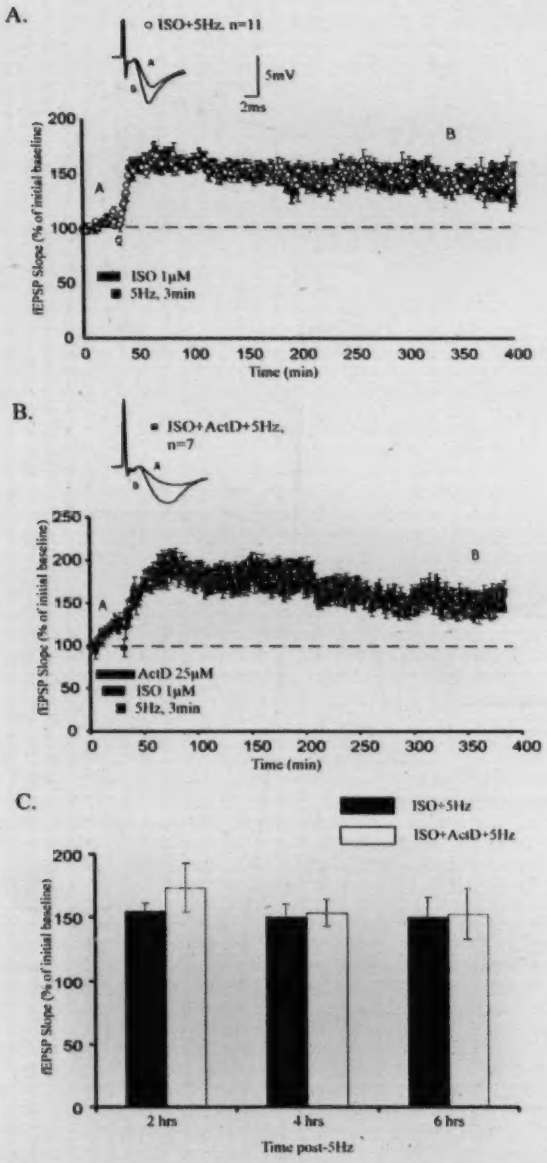


Figure 4.6: L-LTP induced by β-adrenergic receptor activation paired with subthreshold stimulation does not exhibit a late transcriptional component. (A) Application of ISO during 5 Hz stimulation induces L-LTP that lasts for at least 6 hours (open circles). (B) L-LTP is maintained for at least 6 hours when ACT-D is applied during ISO+5 Hz stimulation (filled squares). (C) Summary histogram comparing potentiation levels of slices treated with or without ACT-D at various time points. All sample traces were taken 10 min after commencement of baseline recording and 120 min after stimulation protocol. Calibration: 5 mV, 2 ms.

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# CHAPTER V

## β-Adrenergic Receptor Activation Recruits ERK and mTOR to Initiate Translation and Facilitate Maintenance of Long-Term Potentiation

## 1. Introduction

Neuromodulatory systems tightly control the processing and storage of information to regulate cognitive function in the mammalian brain. Synaptic plasticity is a prime candidate for mediating information storage at the cellular level, and activation of neuromodulatory receptors can modify synaptic strength. One neuromodulator that is strongly implicated in memory and synaptic plasticity is noradrenaline (NA). In the hippocampus, NA acts through β-adrenergic receptors to enhance the retention and recall of information, suggesting a selective role in long-term memory (LTM) (Izquierdo et al., 1998; Ji et al., 2003a; Ji et al., 2003b; Murchison et al., 2004).

*De novo* protein synthesis is a key characteristic of LTM and long-lasting synaptic plasticity (Davis and Squire, 1984; Kandel, 2001; Stanton and Sarvey, 1984). Some evidence suggests that β-adrenergic receptors are involved in plasticity-related protein synthesis. For instance, activation of hippocampal noradrenergic afferents induces a delayed, protein synthesis-dependent long-term potentiation (LTP) of synaptic strength (Walling and Harley, 2004). Similarly, activation of β-adrenergic receptors during weak synaptic activity initiates a protein synthesis-dependent enhancement of LTP (Gelinas and Nguyen, 2005; Huang and Kandel, 1996). The intracellular signaling processes that elicit synthesis of new proteins downstream of the β-adrenergic receptor are unknown.

Protein synthesis in eukaryotes is controlled primarily at the level of translation initiation (Dever, 2002). Recognition of the mRNA and ribosomal recruitment are intricately regulated, rate-limiting steps that involve the initiation factor eIF4E. In the basal state, eIF4E is sequestered by the inhibitory binding protein 4E-BP to prohibit translation (Haghighat et al., 1995; Mader et al., 1995; Pause et al., 1994). Activation of

the mTOR signaling pathway results in hyperphosphorylation of 4E-BP and release of eIF4E (Beretta et al., 1996; Gingras et al., 1998). The binding of eIF4E to another translation factor, eIF4G, forms the translation-promoting eIF4F complex. Phosphorylation of eIF4E by the ERK-dependent kinase Mnk1 increases the rate of translation, although how this phosphorylation modifies the activity of eIF43 is unclear (Duncan et al., 2003; Scheper and Proud, 2002; Wang et al., 1998). Thus, mTOR and ERK signaling cascades are directly involved in the regulation of protein synthesis. Furthermore, mTOR- and ERK-mediated phosphorylation of eIF4E and 4E-BP occurs in response to stimuli that elicit long-lasting LTP and LTM (Banko et al., 2005; Kelleher et al., 2004b; Tang et al., 2002).

β-Adrenergic receptors couple with the guanine-nucleotide-binding regulatory Gs protein to stimulate adenylyl cyclase activity and increase cAMP (Minocherhomjee and Roufogalis, 1982; Raymond, 1995). A main target of cAMP signaling is activation of cAMP-dependent protein kinase (PKA) (Beebe, 1994; Nguyen and Woo, 2003). Here, I investigated the intracellular signaling cascades recruited by β-adrenergic receptor activation to enhance LTP maintenance and initiate protein synthesis in area CA1 of the hippocampus. My results indicate that activation of β-adrenergic receptors induces protein synthesis-dependent LTP via ERK- and mTOR-dependent phosphorylation of translation initiation factors. Furthermore, this intracellular signaling can occur independently of, and in parallel to, the PKA signaling pathway, depending on concurrent patterns of synaptic activity.

## 2. Materials and Methods

### a) Animals

#### *i. C57BL/6 mice*

Female C57BL/6 mice (aged 8-13 weeks) were used for all experiments unless otherwise indicated.

#### *ii. R(AB) mice*

Electrophysiology experiments were also performed on transgenic R(AB) mice (aged 6-10 months) and age-matched wild-type littermate controls. These mice are maintained in a hemizygous state on a C57BL6/J background and were originally derived from two independent genetic lines characterized for R(AB) transgene expression, synaptic physiology, and behaviour (Abel et al., 1997; Clegg et al., 1987). Genotyping was performed by Southern blot using a previously described transgene-specific probe (Abel et al., 1997).

#### *iii. 4E-BP2 knockout mice*

4E-BP2 knockout mice (aged 8-13 weeks) were used for electrophysiology experiments. Heterozygous mice were originally derived on a mixed 129/SvJ and BALB/c background. Congenic C57BL/6 mutant mice were developed using marker-assisted breeding with the assistance of the JAX Genome Services group from the Jackson Laboratory (Bar Harbor, ME). Currently, the 4E-BP2 knockout colony is at N13 on a C57BL/6J background.

All mice were housed under guidelines set forth by the CCAC and IACUC.

b) Electrophysiology

Transverse murine hippocampal slices (400 μm) were obtained following cervical dislocation and decapitation. Electrophysiology experiments were conducted as detailed in Chapter II. LTP was induced with high frequency stimulation (HFS) protocols consisting of either 1 train of 100 Hz (1 s duration) or 4 trains of 100 Hz (1 s duration, 5 min inter-train interval). Theta-train stimulation at 5 Hz for 3 min was also used. fEPSPs were monitored with test stimuli for 120 min after induction of LTP.

c) Drugs

The β-adrenergic receptor agonist isoproterenol [ISO; R(-)-isoproterenol (+)-bitartrate, 1 μM; Sigma-Aldrich Canada, Oakville, ON, Canada] was prepared daily as a 1 mM stock solution in distilled water. The PKA inhibitors KT-5720 (1 μM; Sigma) and Rp-cAMPs (60 μM; Sigma) were prepared as concentrated stock solutions at 1 and 60 mM in DMSO. The ERK inhibitor U0126 (20 μM; Bioshop Canada, Burlington, ON, Canada) and mTOR inhibitor rapamycin (Rap; 1 μM; Bioshop) were dissolved in DMSO to create stock solutions at 20 and 1 mM, respectively. Each drug was diluted in ACSF and bath applied at a perfusion rate of 1-2 mL/min. Experiments were performed in dimmed light conditions due to light sensitivity of drugs.

d) Data Analysis

Using initial slope of the fEPSP as an index of synaptic strength (Johnston and Wu, 1995), levels of LTP were compared between groups 120 min after LTP induction. Student's t-test was used for statistical comparison between two groups, with Welch correction if standard deviations were significantly different between groups. One-way

ANOVA and Tukey-Kramer *post hoc* tests were used when comparing three or more groups. In cases where standard deviations differed between multiple groups, the Kruskal-Wallis test with *post hoc* Dunn's multiple comparisons tests was used. The criterion for significance was $p < 0.05$ in all cases. Data are reported as mean ± standard error (SEM), with n equal to number of slices.

e) Biochemistry

CA1 regions were microdissected from hippocampal slices and prepared for biochemistry. Samples were immunoprecipitated and analyzed with quantitative Western blotting as described in Chapter II. Antibodies used included phospho-ERK antibody (1:5000), phospho-Mnk1 antibody (1:1000), phospho-eIF4E antibody (1:1000), phospho-4E-BP1 antibody (1:500), total eIF4E (1:1000), and eIF4G1 antibody (1:1000). The phospho-4E-BP1 antibody has been shown to cross-react with 4E-BP2, ensuring detection of the predominant neuronally-expressed 4E-BPs (Banko et al., 2005).

f) Immunohistochemistry

After appropriate pharmacological and/or electrical stimulation, hippocampal slices were immediately put in ice-cold 4% paraformaldehyde/0.1% glutaraldehyde in phosphate-buffered saline (PBS) (pH 7.4) and fixed overnight. The slices were then put in 30% sucrose overnight at 4°C and embedded with optimal cutting temperature compound. A sliding microtome was used to cut the slices into 20 µm sections. Free-floating sections were blocked with 10% normal goat serum in PBS/0.7% Triton X-100 (PBS-TX) overnight at 4°C. Sections were then incubated overnight at 4°C with phospho-4E-BP1 (Thr37/46) antibody (1:100). After washing three times with PBS-TX, sections

were incubated for two hours at room temperature with Cy3-conjugated AffiniPure goat anti-rabbit IgG diluted 1:500 in blocking solution. Sections were then washed and mounted onto poly-L-lysine-coated slides. Sections were analyzed and imaged using a Zeiss LSM 510 meta confocal microscope system (Zeiss, Oberkochen, Germany).

**3. Results**

a) β-Adrenergic receptor-dependent enhancement of LTP maintenance requires ERK and mTOR, but not PKA

I used pharmalogical inhibitors to determine the intracellular signaling cascades necessary for the enhanced maintenance of LTP mediated by β-adrenergic receptor activation in area CA1 of the hippocampus. Stimulation with one train of high frequency stimulation (HFS; 1x100 Hz with 1 s duration) induces decremental E-LTP that is protein synthesis independent, and this form of LTP is not substantially disrupted by inhibition of PKA, ERK, or mTOR (Abel et al., 1997; Huang et al., 1996; Tang et al., 2002; Winder et al., 1999). However, pairing this tetanic stimulation with application of a β-adrenergic agonist, ISO, generates long-lasting LTP that requires dendritic protein synthesis (Gelinas and Nguyen, 2005). Given the purported role of the ERK and mTOR signaling cascades in dendritic protein synthesis (Banko et al., 2004; Banko et al., 2006; Kelleher et al., 2004b; Tang et al., 2002), I tested the hypothesis that ERK and mTOR are recruited by β-adrenergic receptor activation to enhance maintenance of LTP generated by this protocol. Application of an ERK inhibitor, U0126 (20 μM), selectively prevented the maintenance of β-adrenergic receptor-dependent LTP. At 120 min after HFS, mean fEPSP slopes were 105 ± 5% for slices treated with U0126, compared to 154 ± 13% for slices treated with ISO+one train HFS alone (**Figure 5.1A:** $p < 0.01$). Application of an mTOR

inhibitor, rapamycin (1 μM), also inhibited LTP maintenance. In this case, mean fEPSP slopes 120 min after HFS were 113 ± 4% in rapamycin treated slices, and 157 ± 12% in slices treated with ISO+one train HFS alone (**Figure 5.1D:** $p < 0.02$).

Classically, β-adrenergic receptor signaling is linked to increases in intracellular cAMP and subsequent activation of PKA (Dunwiddie et al., 1992; Raman et al., 1996; Segal et al., 1981; Winder et al., 1999). However, application of the PKA inhibitors KT5720 (1 μM) and Rp-cAMPs (60 μM) had no significant effect on β-adrenergic receptor-dependent maintenance of LTP. Mean fEPSP slopes 120 min after HFS were 142 ± 8% for slices treated with KT5720, and 148 ± 10% for slices treated with ISO+one train HFS alone (**Figure 5.1B:** $p > 0.5$). Similarly, slices treated with Rp-cAMPs were potentiated to 160 ± 13% 120 min after HFS, compared to 152 ± 8% in slices treated with ISO+one train HFS alone (**Figure 5.1C:** $p > 0.5$). These results suggest that activation of β-adrenergic receptors can enhance hippocampal LTP independently of PKA.

b) Induction of β-adrenergic receptor-dependent LTP results in the coordinated regulation of translation initiation factors Mnk1, eIF4E and 4E-BP

Activation of ERK and mTOR during induction of long-lasting LTP regulates protein synthesis by engaging multiple translation initiation factors (Banko et al., 2004; Banko et al., 2006; Kelleher et al., 2004b; Tang et al., 2002). In addition, it has been shown that the ERK and mTOR signal transduction pathways converge during metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD) to regulate the critical translation initiation factor eIF4E (Banko et al., 2006). Because I found that my novel form of β-adrenergic receptor-facilitated LTP required ERK and

mTOR activation, I sought to determine whether β-adrenergic receptor activation similarly regulated translation initiation factors.

One train of HFS can engage the ERK-Mnk1-eIF4E signal transduction pathway, but it is unknown whether β-adrenergic receptor activation can also recruit this signaling cascade. In collaboration with Dr. Jessica Banko (Vanderbilt University), I found that activation of β-adrenergic receptors with ISO engaged the ERK-Mnk1-eIF4E signal transduction cascade (**Figure 5.2A-G:** phospho-ERK: $p < 0.01$ vs. ACSF; phospho-Mnk1: $p < 0.02$ vs. ACSF; phospho-eIF4E: $p < 0.02$ vs. ACSF). Interestingly, an even greater increase in phospho-immunoreactivity was observed when β-adrenergic receptor activation was paired with one train of HFS (ISO+HFS; phospho-ERK: $p < 0.01$ vs. HFS alone; phospho-Mnk1: $p < 0.05$ vs. HFS alone; phospho-eIF4E: $p < 0.05$ vs. HFS alone). This enhancement was blocked by the ERK inhibitor U0126, suggesting that one consequence of ERK activation during β-adrenergic receptor-enhanced LTP is the recruitment of the translation initiation cascade ERK-Mnk1-eIF4E.

Consistent with my finding that inhibition of PKA did not attenuate the β-adrenergic receptor-dependent LTP enhancement, I found that the PKA inhibitor KT5720 only partially reduced the ISO+HFS-induced ERK-Mnk1-eIF4E phospho-immunoreactivity, leaving a significant increase over ACSF controls (phospho-ERK: $p < 0.01$ vs. ACSF; phospho-Mnk1: $p < 0.05$ vs. ACSF; phospho-eIF4E: $p < 0.01$ vs. ACSF).

In addition to direct phosphorylation by Mnk1, eIF4E is sequestrated by the 4E-BPs. mTOR-dependent hyperphosphorylation of 4E-BP disrupts the 4E-BP-eIF4E inhibitory interaction. Because β-adrenergic receptor-enhanced LTP requires the kinase mTOR, I hypothesized that activation of β-adrenergic receptors with ISO would also

induce 4E-BP phosphorylation. Indeed, I observed an increase in phospho-4E-BP immunoreactivity in hippocampus area CA1 following ISO application (**Figure 5.3A-C:** phospho-4EBP: $p < 0.05$ vs. ACSF). Similar to what I observed for the ERK-Mnk1-eIF4E pathway, I found that pairing ISO and HFS produced an even greater increase in phospho-4E-BP immunoreactivity than either condition alone (phospho-4EBP: $p < 0.05$ vs ISO alone; $p < 0.01$ vs. HFS alone). This enhancement was blocked by the mTOR inhibitor rapamycin, suggesting that mTOR activation during β-adrenergic receptor-enhanced LTP regulates translation initiation by phosphorylation and inhibition of the translational repressor 4E-BP.

In further support of this hypothesis, I found that the ISO+HFS-induced increase in phospho-eIF4E immunoreactivity was also blocked by pretreatment with rapamycin. Importantly, rapamycin was ineffective at reducing the ISO+HFS-induced increase in ERK or Mnk1 phospho-immunoreactivity. mTOR has not been reported to directly phosphorylate eIF4E. ERK-dependent Mnk1 phosphorylation of eIF4E can only occur when eIF4E is bound to eIF4G (Pyronnet et al., 1999). In an effort to define a mechanism for the rapamycin-dependent inhibition of the ISO+HFS induced increase in phospho-eIF4E immunoreactivity, I performed an eIF4G/eIF4E co-immunoprecipitation assay (eIF4G/E co-ip; **Figure 5.3D-E**). In agreement with the results obtained by detecting phospho-specific immunoreactivity of translation factors, pairing ISO with HFS induced a greater increase in the amount of eIF4E that co-precipitated with eIF4G compared to either treatment alone (eIF4G/E co-ip: $p < 0.02$ vs. ISO alone; $p < 0.05$ vs. HFS alone). This increase was blocked by pretreatment with rapamycin, indicating that mTOR regulates eIF4G/eIF4E complex formation. These results suggest that mTOR-

dependent inhibition of 4E-BP releases sequestrated eIF4E and permits formation of the translation initiation complex during β-adrenergic receptor-dependent LTP. Thus, ERK and mTOR signaling pathways converge to regulate translation initiation by phosphorylation and sequestration of eIF4E, respectively.

c) 4E-BP regulates local induction of long-lasting plasticity during β-adrenergic receptor activation

β-Adrenergic receptor-dependent LTP can be induced in isolated CA1 dendrites and in the presence of a transcription inhibitor (Gelinas and Nguyen, 2005). Because the quantitative Western blotting analysis revealed that 4E-BP is a downstream target of β-adrenergic receptor signaling (**Figure 5.3**), I investigated the role of 4E-BP in dendritic regulation of β-adrenergic receptor-dependent LTP by examining the localization of the increased 4E-BP phosphorylation with immunohistochemistry (in collaboration with Dr. Lingfei Hou, Baylor College of Medicine; **Figure 5.4A**). Consistent with the results of the Western blot analysis, application of ISO increased phospho-4E-BP immunoreactivity in CA1 dendrites. Furthermore, pairing ISO with one train of HFS elicited an additive increase in 4E-BP phosphorylation. These results demonstrate that 4E-BP phosphorylation can be detected in dendrites as a result of β-adrenergic receptor activation, and highlight the importance of this translation repressor in regulating LTP. However, the role of 4E-BP could be further solidified by comparing 4E-BP phosphorylation with basal staining of an independent marker in area CA1.

To further examine the role of 4E-BP in β-adrenergic receptor-dependent LTP, I utilized 4E-BP2 knockout mice. These mice lack the predominant brain isoform of 4E-BP and do not demonstrate any observable metabolic, biochemical, or neuroanatomical

abnormalities (Banko et al., 2005). Pairing β-adrenergic receptor activation with one train of HFS elicited persistent LTP in these knockouts that did not differ from LTP generated by the same protocol in normal animals (**Figure 5.4B:** mean fEPSP slopes were 137 ± 2% 120 min after HFS). Interestingly, LTP induced by multiple trains of HFS is impaired in the 4E-BP2 knockouts (Banko et al., 2005). These results suggest that pairing ISO with HFS generates LTP that is mechanistically distinct from LTP induced by multiple trains of HFS at the level of translation initiation regulation. Application of a β-adrenergic agonist alone induced long-lasting LTP in the 4E-BP2 knockouts (**Figure 5.4C:** mean fEPSP slopes were 181 ± 10% 120 min after ISO application). In comparison, ISO application in normal mice results in a small, transient increase in synaptic strength (Gelinas and Nguyen, 2005; Thomas et al., 1996). These results extend findings from a previous study demonstrating that decremental LTP induced by one train of HFS is converted to long-lasting LTP in the 4E-BP2 knockout mice (Banko et al., 2005). Thus, 4E-BP2 is critically involved in gating the induction of long-lasting forms of LTP generated by either electrical stimulation of synapses or neuromodulatory receptor activation.

d) R(AB) mice exhibit intact β-adrenergic receptor-dependent enhancement of LTP maintenance

Because pairing ISO with one train of HFS elicits LTP that requires ERK and mTOR, but not PKA (**Figure 5.1**), I examined the effects of genetic PKA deficiency on β-adrenergic-dependent LTP using R(AB) mice. These mice express an inhibitory form of the RIα regulatory subunit of PKA and demonstrate a 10-fold reduction in basal hippocampal PKA activity (Young et al., 2006). Paired application of ISO and one train

of HFS elicited similarly robust, long-lasting LTP in R(AB)s and wildtype littermates. At 120 min after HFS, mean fEPSP slopes were 193 ± 17% and 185 ± 11% in R(AB)s and wildtype littermates, respectively. In comparison, mean fEPSP slopes 120 min after one train HFS alone were 108 ± 3% in R(AB)s and 110 ± 7% in wildtype littermates (**Figure 5.5A, B**). Consistent with results in C57/BL6 mice, maintenance of β-adrenergic receptor-dependent LTP was prevented by application of U0126 in both R(AB)s and wildtypes. U0126-treated slices had mean fEPSP slopes of 106 ± 3% in R(AB)s and 105 ± 5% in wildtype littermates 120 min after HFS (**Figure 5.5C, D**). Kruskal-Wallis testing followed by Dunn's multiple comparisons detected no significant differences in LTP maintenance or ERK dependence between R(AB)s and corresponding wildtype littermates (**Figure 5.5E**). As such, a genetic deficit of PKA activity does not affect LTP generated by pairing β-adrenergic receptor activation with one train of HFS.

e) Activation of β-adrenergic receptors rescues impairments of LTP maintenance generated by genetic or pharmacologic PKA deficiency

Four trains of HFS (5 min inter-train interval) elicit PKA-dependent L-LTP (Abel et al., 1997). PKA gates LTP maintenance by contributing to transcription initiation in the nucleus (Impey et al., 1996; Nayak et al., 1998). Correspondingly, R(AB) mice display a selective impairment in L-LTP maintenance (Abel et al., 1997; Woo et al., 2002). At 120 min after 4 trains of HFS, mean fEPSP slopes are 116 ± 7% in slices from R(AB) mice, compared to 187 ± 17% in wildtype littermates ($p < 0.01$). If activation of β-adrenergic receptors enhances LTP maintenance independently of PKA, β-adrenergic signaling could rescue L-LTP in R(AB) mice. I tested this hypothesis by applying ISO during 4 train HFS in R(AB)s and wildtype littermates. Whereas ISO did not enhance L-

LTP in wildtype littermates (**Figure 5.6A:** mean fEPSP slopes were 177 ± 10% 120 min after HFS), it rescued L-LTP in R(AB) mice to levels observed in wildtype mice (**Figure 5.6B:** mean fEPSP slopes were 175 ± 14% 120 min after HFS).

Similarly, pharmacologic inhibition of PKA with KT5720 during 4 trains of HFS in C57BL/6 mice resulted in a deficit of L-LTP. At 120 min after HFS, mean fEPSP slopes were 107 ± 9% in KT5720 treated slices, compared to 158 ± 10% in slices treated with 4 train HFS alone (**Figure 5.6D, E:** $p < 0.01$). Consistent with data obtained from the R(AB) mice, pairing ISO application with 4 train HFS rescued the impairment in L-LTP, and restored LTP maintenance (**Figure 5.6E:** mean fEPSP slopes were 157 ± 12% 120 min after HFS). Although ISO application did not affect the amount of potentiation generated by 4 trains of HFS, potentiation levels from slices treated with 4 trains of HFS alone and ISO+4 train HFS were significantly higher than slices additionally treated with KT5720 (**Figure 5.6F**). Taken together, these results suggest that β-adrenergic receptor activation can facilitate maintenance of LTP in parallel with, and independently of, PKA signaling.

f) Induction of β-adrenergic receptor-dependent LTP is inhibited by genetic or pharmacologic PKA deficiency

To assess the contribution of PKA to induction of β-adrenergic-dependent LTP, I examined the effects of PKA deficiency on LTP generated by pairing ISO with low frequency stimulation (LFS). Application of LFS (5 Hz for 3 min) does not persistently alter synaptic strength (Thomas et al., 1996; Woo and Nguyen, 2002). However, co-application of ISO with LFS establishes robust LTP that is dependent on PKA and ERK for its induction (Giovannini et al., 2001; Thomas et al., 1996; Winder et al., 1999).

Consistent with these studies, I found that pairing ISO with LFS in R(AB) mice resulted in blunted induction of LTP. Mean fEPSP slopes were 143 ± 9% 15 min after LFS in R(AB) mice compared to 206 ± 17% in wildtype littermates (**Figure 5.7A, B:** $p < 0.05$). Pharmacologic inhibition of PKA with KT5720 caused a similar decrease in LTP induction, underlining the similarities between LTP phenotypes resulting from genetic or pharmacologic inhibition of PKA (**Figure 5.7C**). Overall, these data suggest that PKA plays distinct roles in the induction and maintenance of β-adrenergic receptor-dependent LTP depending on the concurrent patterns of electrical stimulation used.

**4. Discussion**

Because persistent LTP requires synthesis of new proteins, regulation of translation can gate the establishment of long-lasting plasticity (Deadwyler et al., 1987; Frey et al., 1988; Huang et al., 1996; Kandel, 2001; Stanton and Sarvey, 1984). My results reveal that β-adrenergic receptors can couple to protein synthesis machinery via ERK- and mTOR-dependent engagement of translation initiation. This link provides a molecular mechanism for the enhanced maintenance of LTP generated by activating β-adrenergic receptors during LTP induction. Moreover, the β-adrenergic receptor-dependent facilitation of translation and LTP stability does not require PKA, suggesting that independent signaling pathways are activated downstream of this receptor to mediate distinct intracellular processes.

ERK and mTOR signaling cascades are implicated in the translational regulation of various forms of synaptic plasticity (Banko et al., 2006; Kelleher et al., 2004a; Kelleher et al., 2004b; Tang et al., 2002). In this study, I demonstrated that inhibition of

ERK or mTOR prevents the maintenance, but not the induction, of LTP generated by pairing β-adrenergic receptor activation with one train of HFS. I also directly established that β-adrenergic receptor-dependent LTP recruits cap-dependent translation by revealing increases in translation factor phosphorylation and eIF4F complex formation during induction of this long-lasting LTP. These increases were completely attenuated by inhibition of ERK or mTOR. Consistent with previous work indicating that activation of β-adrenergic receptors elicits LTP that does not require somatic transcription (Gelinas and Nguyen, 2005), translation factor phosphorylation was observed in hippocampal dendrites. Taken together, my results suggest that ERK and mTOR signaling downstream of the β-adrenergic receptor locally increases protein synthesis to stabilize LTP.

My results indicate that signals from neuromodulatory and neurotransmitter receptors are integrated at the level of translation initiation. Application of a β-adrenergic agonist to area CA1 of the hippocampus elicits a transient enhancement of synaptic strength, and a modest increase in ERK- and mTOR-dependent translation factor phosphorylation. One train of HFS generates decremental, protein synthesis-independent LTP and a modest increase in translation factor phosphorylation. Interestingly, when β-adrenergic receptors are activated during one train of HFS, translation factor phosphorylation is substantially increased, resulting in protein synthesis-dependent LTP. The phosphorylation state of key translation initiation factors, such as eIF4E and 4E-BP, therefore reflects the integration of diverse intracellular signals and provides a mechanism by which activation of neuromodulatory receptors can influence the induction and maintenance of LTP. This additive integration process further suggests that a critical

threshold of translation initiation must be reached to generate long-lasting, protein synthesis-dependent plasticity. In support of this notion, genetic facilitation of translation converts decremental LTP to long-lasting LTP (Banko et al., 2005; Barco et al., 2002). For example, mice that lack the inhibitory translation regulator 4E-BP2 have increased basal levels of translation initiation complex formation. Application of one train of HFS to hippocampal slices of these mice results in stable, protein synthesis-dependent LTP (Banko et al., 2005). The current data extend these findings by demonstrating that application of a β-adrenergic agonist alone similarly induces long-lasting LTP in these mice. Thus, recruitment of protein synthesis during long-lasting synaptic plasticity is heavily influenced by the degree to which synaptic stimulation engages translation initiation machinery, rather than the form of this stimulation.

However, translation initiation requires the coordinated activity of multiple intracellular signaling pathways. ERK and mTOR signaling cascades appear to independently converge at regulation of eIF4E during β-adrenergic receptor-dependent LTP. Inhibition of mTOR does not affect ERK or Mnk1 phosphorylation, although it decreases eIF4E phosphorylation. Because eIF4E is only phosphorylated by Mnk1 when it is bound to eIF4G (Gingras et al., 1999), blocking mTOR could prevent ERK-dependent eIF4E phosphorylation by inhibiting eIF4E association with eIF4G. In support of this hypothesis, the mTOR inhibitor rapamycin decreases the amount of eIF4E that co-immunoprecipitates with eIF4G. Given that inactivation of either the ERK or mTOR pathway blocks establishment of long-lasting β-adrenergic receptor-dependent LTP, this dual regulation of eIF4E ensures that translation is initiated only during concurrent ERK and mTOR signaling. Conjoint ERK and mTOR signaling is also required for mGluR-

LTD, another form of plasticity that induces local protein synthesis (Banko et al., 2006; Huber et al., 2000).

Modest levels of translation factor phosphorylation may play a role in synaptic tagging. One train of HFS does not induce protein synthesis, but it does endow synapses with the ability to 'capture' protein products generated by stronger stimulation applied to nearby synapses (Frey and Morris, 1997; Martin and Kosik, 2002). It is possible that activation of translation factors following one train of HFS contributes to tagging the synapse for future modifications (Banko et al., 2005). Because activation of β-adrenergic receptors alone modestly stimulates translation factors, this type of neural activity may also tag synapses. Further experimentation is necessary to investigate this idea.

The β-adrenergic receptor couples to adenylyl cyclase via the Gs form of G-protein (Minocherhomjee and Roufogalis, 1982; Raymond, 1995). As such, β-adrenergic receptor signaling is mediated by increases in cAMP. PKA is a prime target of cAMP signaling, and is required for some forms of long-lasting LTP (Abel et al., 1997; Frey et al., 1993). I found that activation of β-adrenergic receptors during one train of HFS does not require PKA to facilitate maintenance of LTP, and genetic deficiency of PKA activity does not block this β-adrenergic receptor-dependent LTP. Furthermore, inhibition of PKA only partially attenuated activation of the ERK-Mnk1-eIF4E pathway that resulted from pairing ISO with HFS. These results suggest that PKA does not importantly contribute to the translational control of LTP downstream of the β-adrenergic receptor.

Conversely, β-adrenergic receptor activation can recruit PKA to modify channel function and facilitate some forms of LTP (Raman et al., 1996; Thomas et al., 1996; Winder et al., 1999). We confirmed that inhibition of PKA blunts the induction of LTP

generated by pairing a β-adrenergic agonist with LFS. This form of LTP is also impaired by ERK inhibition (Giovannini et al., 2001; Winder et al., 1999), and I further demonstrated that blockade of mTOR completely inhibits this LTP (data not shown here). Whereas concurrent patterns of synaptic activity heavily influence whether PKA is recruited downstream of the β-adrenergic receptor, β-adrenergic receptor-dependent LTP requires ERK and mTOR regardless of whether LFS or HFS is utilized for induction. Furthermore, activation of β-adrenergic receptors can rescue impairments of L-LTP caused by pharmacologic or genetic deficiencies of PKA activity. Taken together, these results indicate that β-adrenergic receptor activation can differentially recruit PKA in response to specific patterns of synaptic activity, and that PKA signaling can operate in conjunction with, but parallel to, ERK and mTOR signaling.

Although cAMP can activate ERK via PKA and a secondary signaling cascade involving Rap1 and B-raf (Waltereit and Weller, 2003), PKA-independent activation of ERK has also been demonstrated. For instance, in neuronal cell lines, activation of Gs-coupled 5-HT7A receptors results in stimulation of cAMP-regulated guanine exchange factors (cAMP-GEFs, also known as Epac) and subsequent augmentation of ERK activity (Lin et al., 2003a). Epac can also activate PI3K in certain systems (Johnson-Farley et al., 2005). Thus, it is reasonable to hypothesize that direct coupling of cAMP to ERK and mTOR signaling cascades can occur downstream of the β-adrenergic receptor.

In summary, my observations demonstrate an intricate biochemical signaling mechanism by which activation of β-adrenergic receptors can modulate the persistence of synaptic plasticity. I also identify a novel β-adrenergic receptor-dependent, but PKA-independent, signaling pathway that specifically contributes to translational regulation of

plasticity. Determining how activation of neuromodulatory receptors, such as the β-adrenergic receptor, contributes to long-lasting plasticity should provide insight into how information is selected for long-term storage in the mammalian brain. Given the importance of neuromodulatory systems in cognitive function, such insights may also identify novel therapies for human disorders of memory, including Alzheimer's disease and posttraumatic stress disorder.

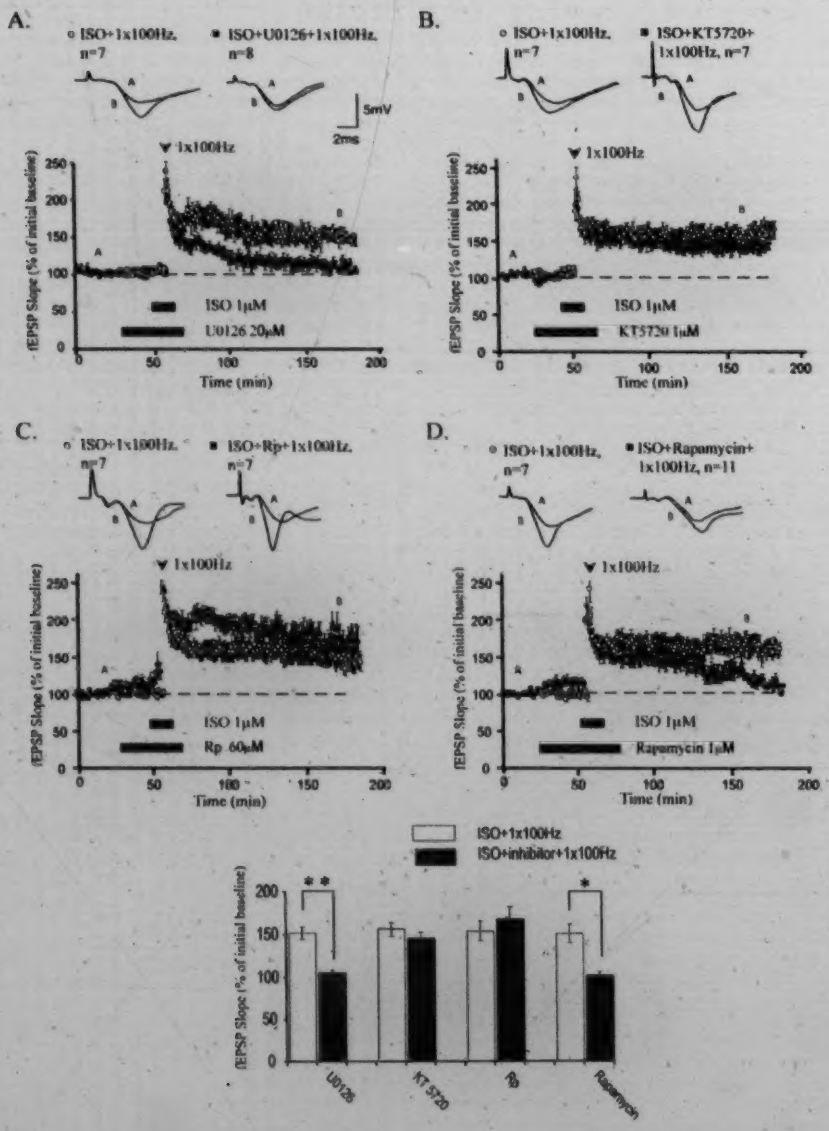



Figure 5.1: β-Adrenergic receptor-dependent enhancement of LTP maintenance requires ERK and mTOR, but not PKA. (A) Application of U0126 causes LTP generated by pairing one train HFS with ISO to decay to levels significantly below U0126-free controls. (B) Application of KT5720 does not inhibit maintenance of LTP elicited by pairing ISO with HFS. (C) Application of Rp does not inhibit maintenance of LTP elicited by pairing ISO with HFS. (D) Application of Rap causes LTP generated by pairing one train HFS with ISO to decay to levels significantly below Rap-free controls. (D) Summary histogram comparing effects of different inhibitors on LTP maintenance 120 min after HFS (*$p<0.05$, **$p<0.01$). All sample traces were taken 10 min after commencement of baseline recording and 120 min after HFS. Calibration: 5 mV, 2 ms.

Figure 5.2: Induction of β-adrenergic receptor-dependent LTP results in ERK-dependent regulation of translation initiation factors Mnk1 and eIF4E. (A) Representative Western blots demonstrating that β-adrenergic receptor activation produced an increase in the phosphorylation of ERK, Mnk1 and eIF4E. A greater increase in phospho-specific immunoreactivity was observed when β-adrenergic receptor activation was paired with HFS. (B-G) Quantification of the phospho-immunoreactivity in A. (B) Pairing ISO with one train of HFS resulted in significantly more phospho-ERK immunoreactivity than either ISO or HFS alone. This increase was abrogated by pretreatment with U0126, partially attenuated by pretreatment with KT5720, and insensitive to pretreatment with rapamycin (rap). (C) Only U0126 significantly reduced basal levels of phospho-ERK immunoreactivity. (D) Similar to ERK, pairing ISO with one train of HFS resulted in significantly more phospho-Mnk1 immunoreactivity than either ISO or HFS alone. This increase was abrogated by pretreatment with U0126, partially attenuated by pretreatment with KT5720 and insensitive to pretreatment with rap. (E) The inhibitors alone were insufficient to significantly reduce basal phospho-Mnk1 immunoreactivity. (F) Pairing ISO with one train of HFS resulted in significantly more phospho-eIF4E immunoreactivity than either ISO or HFS alone. This increase was abrogated by pretreatment with either U0126 or rap and partially attenuated by pretreatment with KT5720. (G) Only rap significantly reduced basal levels of phospho-eIF4E immunoreactivity. '*' denotes significant difference from ACSF control mean. '#' denotes significance difference between bracketed conditions. For ACSF, ISO, HFS and ISO+HFS, n=7. For U0126, n=3. For all other conditions, n=4.

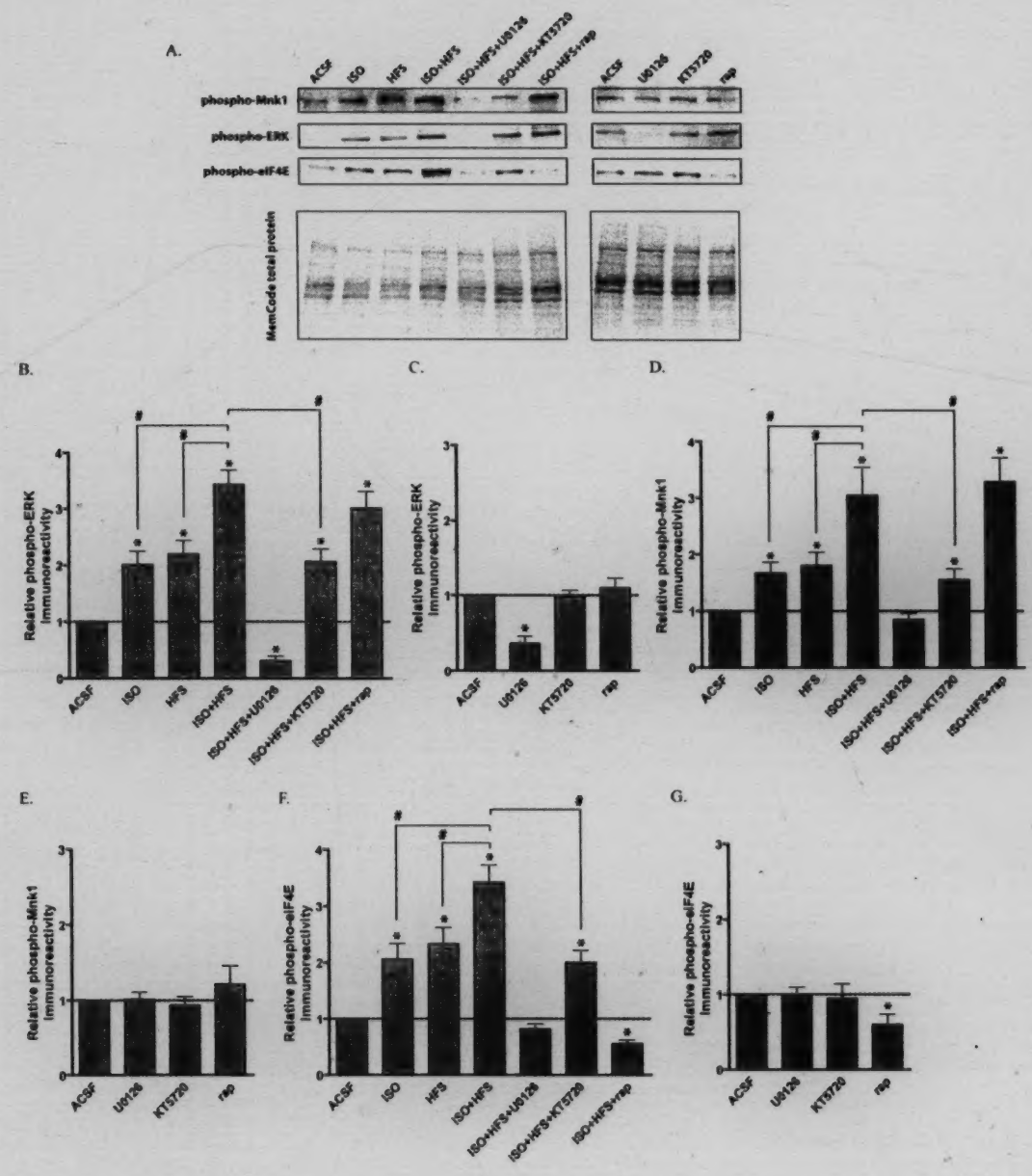
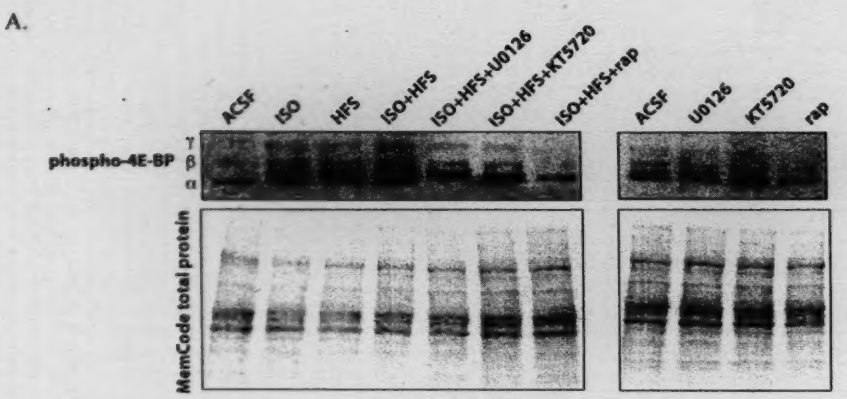
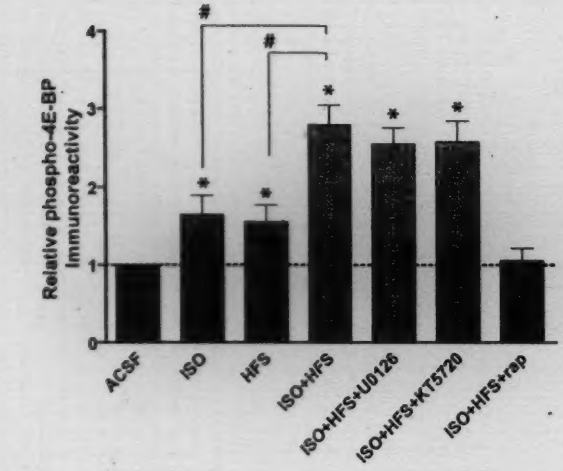
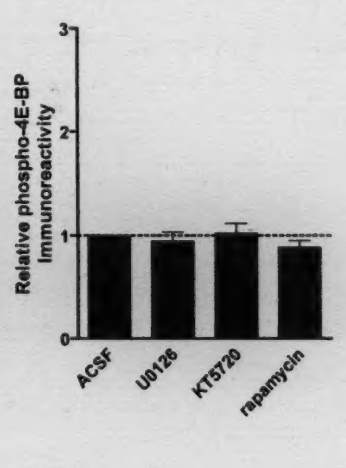
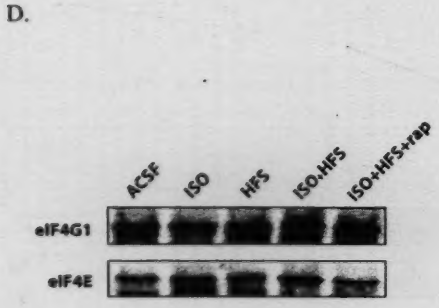

A.
ACSF
ISO
HFS
ISO+HFS
ISO+HFS+U0126
ISO+HFS+KT5720
ISO+HFS+rap
ACSF
U0126
KT5720
rap
phospho-Mnk1
phospho-ERK
phospho-eIF4E
MemCode total protein
B.
C.
D.
Relative phospho-ERK Immunoreactivity
Relative phospho-ERK Immunoreactivity
Relative phospho-Mnk1 Immunoreactivity
E.
F.
G.
Relative phospho-Mnk1 Immunoreactivity
Relative phospho-eIF4E Immunoreactivity
Relative phospho-eIF4E Immunoreactivity

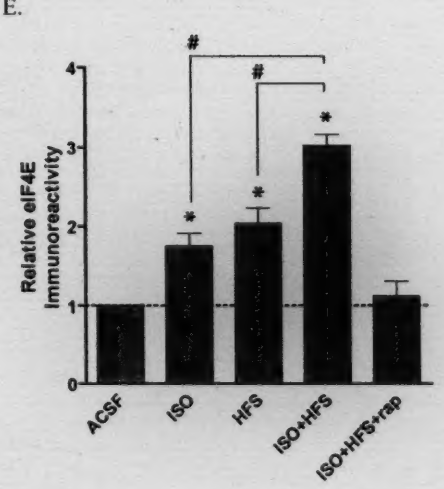
Figure 5.3: Induction of β-adrenergic receptor-dependent LTP results in mTOR-dependent regulation of translation initiation factors 4E-BP and eIF4E. (A) Representative Western blots demonstrating that β-adrenergic receptor activation produced an increase in the phosphorylation of 4E-BP. A greater increase in phospho-specific immunoreactivity was observed when β-adrenergic receptor activation was paired with HFS. (B-C) Quantification of the phospho-specific immunoreactivity in A. (B) Pairing ISO with one train of HFS resulted in significantly more phospho-4E-BP immunoreactivity (α,β, and γ phosphorylation states analyzed together) than either ISO or HFS alone. This increase was abrogated by pretreatment with rap and insensitive to pretreatment with either U0126 or KT5720. (C) Although rap attenuated basal phospho-4E-BP immunoreactivity, none of the inhibitors alone could reduce basal phospho-4E-BP immunoreactivity significantly. (D) Representative Western blots demonstrating that β-adrenergic receptor activation produced an increase in the quantity of total eIF4E that co-precipitates with eIF4G using an anti-eIF4G antibody. A greater increase in total eIF4E immunoreactivity was observed when β-adrenergic receptor activation was paired with HFS and this increase was abolished by pretreatment with rap. (E) Quantification of the total eIF4E immunoreactivity co-immunoprecipitated with the eIF4G antibody in D. '*' denotes significant difference from ACSF control mean. '#' denotes significant difference between bracketed conditions. For ACSF, ISO, HFS and ISO+HFS, n=7. For U0126, n=3. For all other conditions, n=4.


A.
ACSF
ISO
HFS
ISO+HFS
ISO+HFS+U0126
ISO+HFS+KT5720
ISO+HFS+rap
ACSF
U0126
KT5720
rap
phospho-4E-BP
γ
β
α
MemCode total protein


B.
Relative phospho-4E-BP Immunoreactivity
ACSF
ISO
HFS
ISO+HFS
ISO+HFS+U0126
ISO+HFS+KT5720
ISO+HFS+rap

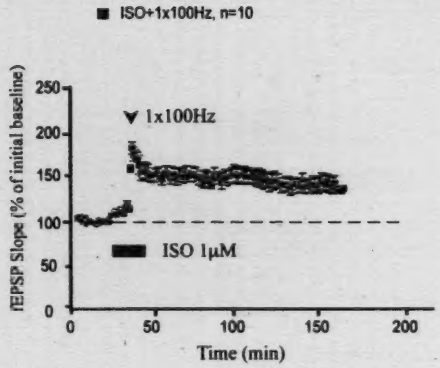

C.
Relative phospho-4E-BP Immunoreactivity
ACSF
U0126
KT5720
rapamycin


D.
ACSF
ISO
HFS
ISO+HFS
ISO+HFS+rap
eIF4G1
eIF4E


E.
Relative eIF4E Immunoreactivity
ACSF
ISO
HFS
ISO+HFS
ISO+HFS+rap

A.

B.




C.

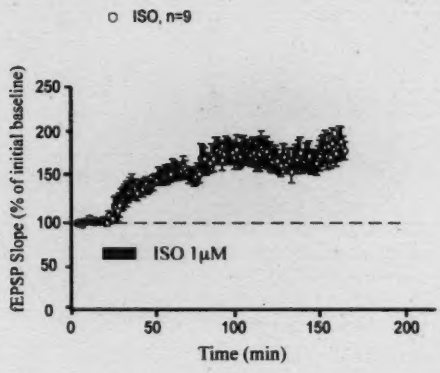



Figure 5.4: 4E-BP regulates local induction of long-lasting plasticity during β-adrenergic receptor activation. (A) Representative micrographs depicting changes in phospho-4E-BP immunoreactivity in control slices (ctrl), and slices treated with ISO, one train HFS, or ISO + one train HFS. A greater increase in phospho-4E-BP immunoreactivity was observed in area CA1 when ISO application was paired with HFS than when ISO or HFS was applied alone. Similar results were obtained for ctrl, ISO, ISO+HFS in 5 independent experiments. Similar results were obtained for HFS in 3 independent experiments. Stratum pyramidale (s.p.) and stratum radiatum (s.r.) are indicated. Electrode placement for original electrophysiology is indicated by '#'. (B) In 4E-BP2 knockout mice, pairing ISO with one train HFS induces long-lasting LTP. (C) In 4E-BP2 knockout mice, application of ISO alone also induces long-lasting LTP.

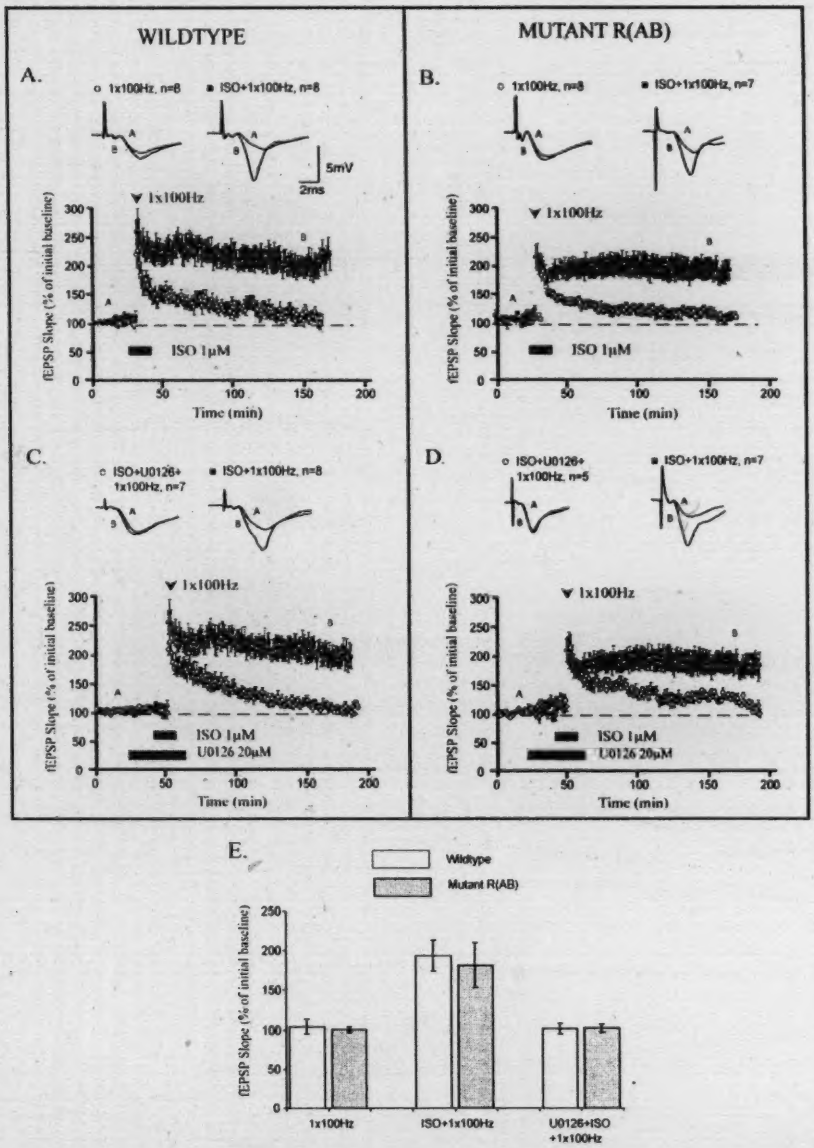



Figure 5.5: R(AB) mice exhibit intact β-adrenergic receptor-dependent enhancement of LTP maintenance. (A) In wildtype mice, application of ISO during one train HFS induces long-lasting LTP, whereas one train HFS alone induces decremental LTP. (B) Application of ISO during one train HFS in mutant mice similarly induces long-lasting LTP. (C) Application of U0126 causes LTP generated by pairing one train HFS with ISO in wildtype mice to decay to levels significantly below U0126-free controls. (D) Application of U0126 causes LTP generated by pairing one train HFS with ISO in mutant mice to decay to levels significantly below U0126-free controls. (E) Summary histogram for these experiments comparing levels of potentiation 120 min after HFS. All sample traces were taken 10 min after commencement of baseline recording and 120 min after HFS. Calibration: 5 mV, 2 ms.

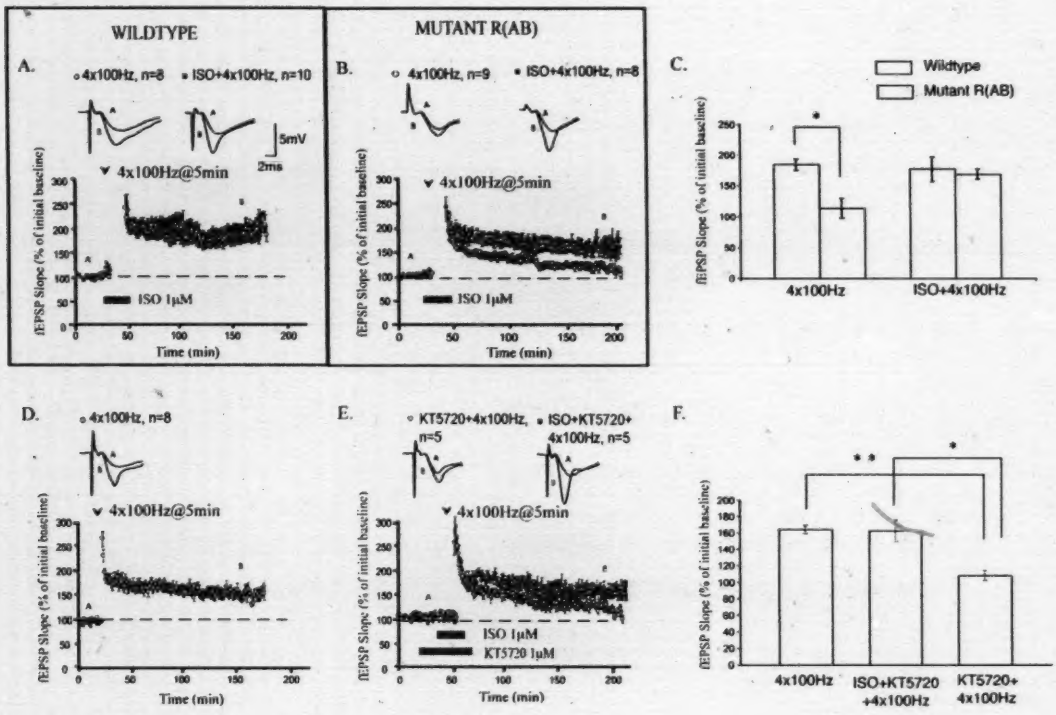



Figure 5.6: Activation of β-adrenergic receptors rescues impairments of LTP maintenance generated by genetic or pharmacologic PKA deficiency. (A) In wildtype mice, application of ISO does not alter LTP generated by 4 trains of HFS. (B) In mutant mice, maintenance of LTP generated by 4 trains of HFS is impaired. Application of ISO significantly enhances the maintenance of this LTP. (C) Summary histogram for these experiments. (D) 4 trains of HFS generate long-lasting LTP in normal mice. (E) Application of KT5720 causes LTP elicited by 4 trains of HFS to decay to levels significantly below KT5720-free controls. Application of ISO enhances the maintenance of this LTP. (F) Summary histogram for these experiments (*$p<0.05$, **$p<0.01$). All sample traces were taken 10 min after commencement of baseline recording and 120 min after HFS. Calibration: 5 mV, 2 ms.

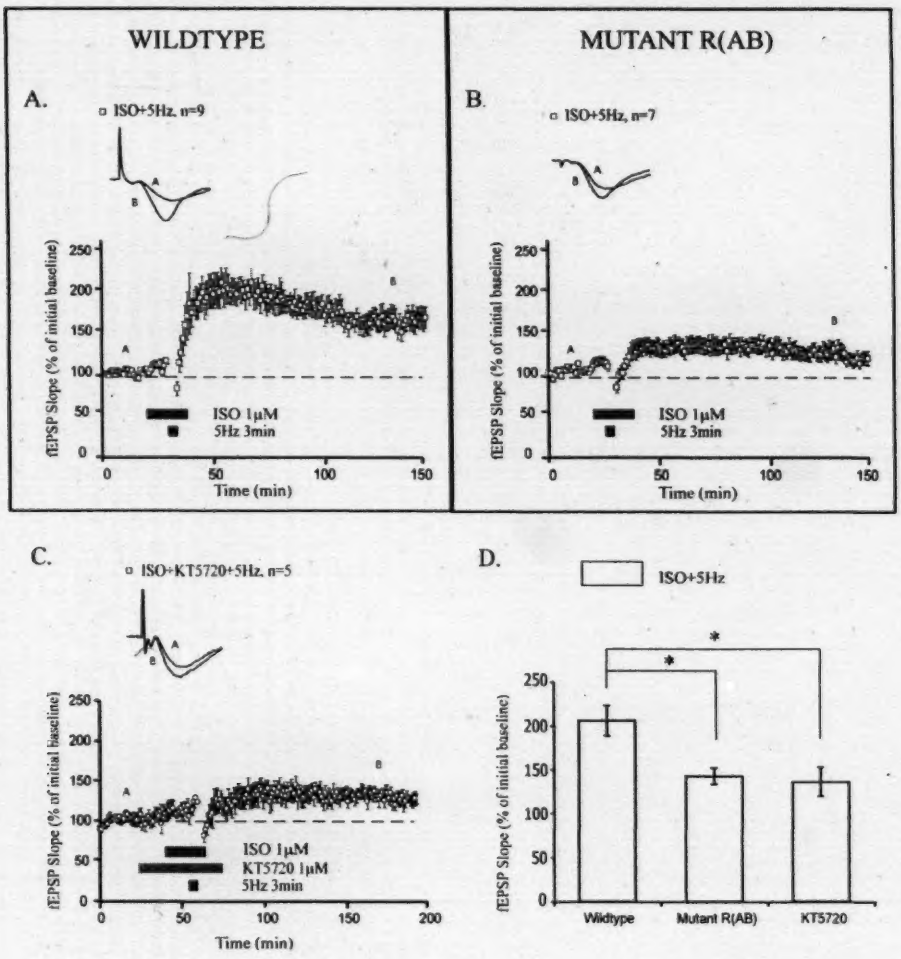



Figure 5.7: Induction of β-adrenergic receptor-dependent LTP is inhibited by genetic or pharmacologic PKA deficiency. (A) In wildtype mice, pairing ISO with LFS generates long-lasting LTP. (B) In mutant mice, induction of LTP generated by pairing ISO with LFS is impaired. (C) Similarly, application of KT5720 blocks induction of LTP generated by pairing ISO with LFS. (D) Summary histogram for these experiments (*$p<0.05$). All sample traces were taken 10 min after commencement of baseline recording and 120 min after LFS. Calibration: 5 mV, 2 ms.

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# CHAPTER VI

## Activation of cAMP-Regulated Guanine Nucleotide Exchange Factor Engages Protein Synthesis to Enhance the Maintenance of Long-Term Potentiation

## 1. Introduction

Hippocampal area CA1 is crucial for long-term memory formation in mice and humans (Tsien et al., 1996; Zola-Morgan et al., 1986). CA1 synapses can express persistent alterations in synaptic strength that are thought to underlie this information storage (Abraham et al., 2002; Bliss and Collingridge, 1993; Lynch, 2004; Moser et al., 1998). Increases (long-term potentiation, LTP) or decreases (long-term depression, LTD) in synaptic strength are mediated by complex interactions of intracellular signaling molecules (Braunewell and Manahan-Vaughan, 2001; Sanes and Lichtman, 1999). 3', 5'-Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger for intracellular neural communication. Adenylyl cyclases generate cAMP from ATP when activated by stimulatory guanine nucleotide-binding regulatory proteins (Gs-proteins) and calcium, thereby integrating signals from several key receptors (Ferguson and Storm, 2004). cAMP is also strongly implicated in hippocampal synaptic plasticity and memory. For instance, genetic elimination of calcium/calmodulin-stimulated adenylyl cyclases (AC1 and AC8) blocks late phase-LTP (L-LTP) and long-term memory (LTM) for contextual and passive avoidance conditioning (Wong et al., 1999). Similarly, stimulation of cAMP signaling in area CA1 initiates L-LTP (Frey et al., 1993), and stimulation of adenylyl cyclase modulates the kinetics of ionotropic glutamate receptor channels (Greengard et al., 1991; Raman et al., 1996; Wang et al., 1991).

Although cAMP-dependent protein kinase (PKA) is thought to be the prime downstream effector of cAMP, the existence of cAMP-regulated guanine exchange factors (GEFs) known as Epacs (exchange proteins directly activated by cAMP) diversifies the signaling potential of cAMP. Epacs are expressed in the nervous system

(Kawasaki et al., 1998), and they bind cAMP to activate a GTP-ase, Rap, in a PKA-independent fashion (de Rooij et al., 1998). Because Rap can interact with the ERK cascade, Epacs are associated with modulation of ERK-dependent processes in various systems (Johnson-Farley et al., 2005; Keiper et al., 2004; Lin et al., 2003a; Traver et al., 2006). In the hipocampus, ERK is required for many forms of synaptic plasticity (Sweatt, 2004). In particular, the ERK signaling cascade regulates protein synthesis during long-lasting LTP and LTD via phosphorylation of translation initiation factors (Banko et al., 2004; Banko et al., 2006; Kelleher et al., 2004b; Schmitt et al., 2005). Given the importance of cAMP and ERK signaling in the hippocampus, it is possible that activation of Epac is a physiologically relevant occurrence in this brain region as well. However, it is not known whether activation of Epac can influence hippocampal synaptic plasticity.

I show here that acute perfusion of mouse hippocampal slices with a specific agonist of Epac enhances the maintenance of LTP without affecting basal synaptic transmission or initial LTP induction. This enhancement of LTP stability requires protein synthesis and activation of ERK, but not transcription. My data reveal, for the first time in the literature, that activation of Epac facilitates LTP in a hippocampal subregion that is known to be important for the formation of long-term memories (Zola-Morgan et al., 1986).

**2. Materials and Methods**

a) Electrophysiology

8-13 week old female C57/BL6 mice (Charles River, Montreal, Canada) were sacrificed and decapitated. Transverse hippocampal slices (400 μm) were obtained and electrophysiology experiments were conducted as detailed in Chapter II. Input-output (I/O) data were collected by varying the intensities of 7 stimuli applied to area CA1. Paired-pulse facilitation (PPF) was examined by applying two pulses at interpulse intervals of 50, 100, 150 and 200 ms. LTP was induced with one train of high-frequency stimulation (100 Hz for 1 s duration at 40% of maximal fEPSP amplitude). Theta-train stimulation at 5 Hz for 3 min was also applied.

b) Drugs

A specific Epac agonist, 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT, 100 μM; Axxora, San Diego, CA; (Enserink et al., 2002) was prepared as a concentrated stock solution at 50 mM in DMSO. The β-adrenergic receptor agonist isoproterenol [ISO; R(-)-isoproterenol (+)-bitartrate, 1 μM; Sigma-Aldrich Canada, Oakville, ON, Canada] was prepared daily as a 1 mM stock solution in distilled water. A translation inhibitor, emetine (20 μM; Sigma-Aldrich Canada, Oakville, ON, Canada), and a transcription inhibitor, actinomycin D (ActD, 25 μM; Bioshop Canada, Burlington, ON, Canada) were prepared as concentrated stock solutions at 20 mM in distilled water and 25 mM in DMSO, respectively. The concentrations of these inhibitors have been shown to be effective for blocking macromolecular synthesis in hippocampal slices (Nguyen et al., 1994; Stanton and Sarvey, 1984). An ERK inhibitor, U0126, (20 μM; Bioshop Canada) was prepared as a concentrated stock solution at 20 mM in DMSO, and a PKA inhibitor KT-5720 (1 μM; Sigma) was prepared as a concentrated stock solution at 1 mM in DMSO. Each drug was diluted to its final concentration in ACSF and bath applied. The

final concentration of DMSO did not affect basal synaptic transmission or LTP (data not shown). Sodium okadaic acid (OA, 1 μM; Sigma) was prepared as a concentrated 1 mM stock solution in distilled water. Hippocampal slices were incubated in OA for 90-180 min before commencing experiments. Experiments were performed blind and interleaved.

c) Data Analysis

PPF, I/O and LTP were assessed in the Schaeffer collateral pathway of hippocampal slices. For PPF analysis, Student's t-test was used to compare percentage facilitation between groups at each interpulse interval. I/O analysis was conducted by plotting presynaptic fibre volley amplitudes and fEPSP slopes, then determining linear regression values. Linear regressions between groups were compared using Student's t-test. For LTP experiments, Student's t-test was used to statistically compare means of fEPSP slopes between two groups 120 min after experimental protocol commenced. Welch correction was applied when standard deviations of compared groups differed significantly. The Kruskal-Wallis test followed by Dunn's multiple comparisons was used to compare means of three or more groups. The significance criterion was $p < 0.05$ in all cases. All values are shown as mean ± standard error (SEM), with n = number of slices.

**3. Results**

a) 8-pCPT does not alter basal synaptic properties in area CA1 of the hippocampus

As a preliminary step toward characterizing the effects of 8-pCPT in area CA1 of the hippocampus, I examined basal synaptic function. The relationship between

presynaptic fibre volley and fEPSP slope was determined over a range of stimulus intensities as a measure of synaptic responsiveness. I observed no differences between these input-output (I/O) properties in 8-pCPT-treated slices and ACSF-treated control slices (**Figure 6.1A:** 8-pCPT, $y = 4.9x$, $R^2 = 0.80$; Control, $y = 4.7x$, $R^2 = 0.77$; $p > 0.2$), indicating that 8-pCPT does not alter basal synaptic transmission.

Paired-pulse facilitation (PPF), a widely used method to infer changes in probability of transmitter release, was similarly unaltered by application of 8-pCPT. No significant differences in PPF were observed between ACSF-treated control slices and 8-pCPT-treated slices at 50, 100, 150, or 200 ms inter-pulse intervals (**Figure 6.1B**). As such, application of 8-pCPT does not alter basal synaptic properties in hippocampal area CA1.

b) 8-pCPT enhances LTP maintenance, without affecting LTP induction or basal synaptic transmission

To address whether activation of Epac by 8-pCPT alters long-lasting forms of plasticity, I investigated its effects on LTP induction and maintenance. Firstly, application of 8-pCPT to hippocampal slices during baseline 'test' stimuli does not affect synaptic strength during or after drug application (**Figure 6.2A:** mean fEPSP slopes were 98.0 ± 6.6% and 102.8 ± 4.3% for ACSF and 8-pCPT-treated slices respectively, 15 min after drug application; $p > 0.5$). I next monitored the outcome of applying 8-pCPT during LFS (5 Hz for 3 min). This pattern of LFS alone does not persistently alter synaptic strength, but can permit induction of LTP or LTD depending on the metaplastic 'state' of the synapses (Azad et al., 2004; Liang et al., 2002; Thomas et al., 1996). Synaptic responses from slices treated with 8-pCPT did not differ compared to those elicited from ACSF control slices (**Figure 6.2B:** mean fEPSP slopes were 101.1 ± 5.6% for ACSF-treated slices and 106.7 ± 10.5% for 8-pCPT-treated slices 120 min after LFS).

To further investigate possible effects of 8-pCPT on LTP induction and maintenance, I used a weak tetanus protocol. In area CA1 of the mouse hippocampus, one train of high frequency electrical stimulation (HFS) at 100 Hz (1 s duration) induces an early phase of LTP (E-LTP) that decays to baseline within 2 hours (Duffy et al., 2001; Huang et al., 1996). Applying 8-pCPT during this weak tetanus did not change the initial amount of potentiation generated (**Figure 6.2C**) (mean fEPSP slopes were 219.5 ± 13.4% and 224.6 ± 14.9% for ACSF and 8-pCPT-treated slices respectively, 2 min after 1x100 Hz stimulation; $p > 0.5$). These data suggest that activation of Epac does not augment the capacity of electrical stimuli to induce LTP. However, pharmacologic activation of Epac with 8-pCPT during weak tetanus does enhance the maintenance of LTP (**Figure 6.2C:** mean fEPSP slopes were potentiated to 163.6 ± 13.1% 120 min after 1x100 Hz; $p < 0.01$ compared to 104.3 ± 9.0% for ACSF controls). Therefore, Epac activation increases the stability of LTP generated by one train of HFS without affecting its induction.

c) LTP elicited by pairing HFS with 8-pCPT is stable for at least six hours

To determine whether application of 8-pCPT during HFS persistently increases synaptic strength, I examined levels of potentiation at extended time points after HFS. Mean fEPSP slopes from 8-pCPT-treated slices were consistently enhanced relative to ACSF-treated slices two, four and six hours after HFS (**Figure 6.3:** mean fEPSP slopes were 156.9 ± 12.6% in 8-pCPT-treated slices 360 min after HFS; $p < 0.05$ compared to 104.7 ± 7.5% in ACSF-treated slices). As such, Epac activation generates long-lasting, stable alterations in synaptic strength.

d) LTP elicited by pairing HFS with 8-pCPT requires protein synthesis and ERK, but not transcription

Long-term stability of synaptic plasticity is associated with *de novo* protein synthesis. The production of new proteins is a key feature of long-lasting LTP (Deadwyler et al., 1987; Frey et al., 1988; Kandel, 2001; Nguyen and Kandel, 1996; Stanton and Sarvey, 1984). I tested the hypothesis that activating Epac generates stable, protein synthesis-dependent LTP. One train of HFS given to area CA1 of the hippocampus elicits decremental LTP that is independent of protein synthesis. As such, bath application of a translation inhibitor, emetine (20 μM), had no effect on this form of LTP (**Figure 6.4A**). However, emetine blocked the enhancement of LTP maintenance induced by pairing 8-pCPT and one train of HFS (**Figure 6.4A:** mean fEPSP slopes were 109.0 ± 6.8% for 8-pCPT-treated slices 120 min after 1x100 Hz stimulation; $p > 0.5$ compared to 104.7 ± 11.0% for ACSF controls). Activation of Epac during HFS therefore recruits protein synthesis to facilitate stability of LTP.

I also used a transcription inhibitor, actinomycin D (25 μM), to explore the transcriptional dependence of LTP enhancement resulting from Epac activation. Bath application of actinomycin D did not affect the stability of this form of LTP. After 120 min, mean fEPSP slopes of 8-pCPT-treated slices were significantly potentiated compared to mean fEPSP slopes from ACSF-treated slices (**Figure 6.4B:** mean fEPSP slopes were 150.9 ± 13.4% for 8-pCPT-treated slices 120 min after 1x100 Hz stimulation; $p < 0.02$ compared to 104.7 ± 10.4% for ACSF controls). Pairing 8-pCPT with HFS therefore induces LTP that requires protein synthesis, but not transcription.

The ERK-MAPK signaling pathway is crucial for some forms of synaptic plasticity (Sweatt, 2004; Thomas and Huganir, 2004) and is specifically implicated in translational control of plasticity (Banko et al., 2004; Banko et al., 2006; Gallagher et al.,

2004; Kelleher et al., 2004b). Consequently, I examined the effect of ERK inhibition on my Epac-enhanced LTP. Because LTP induced by 1x100 Hz does not require ERK signaling (Winder et al., 1999), any effects on this LTP can be attributed to Epac activation. Bath application of the ERK inhibitor U0126 (20 μM) selectively blocked the 8-pCPT-dependent maintenance of LTP (**Figure 6.4C:** mean fEPSP slopes were 107.9 ± 7.1% for 8-pCPT-treated slices 120 min after 1x100 Hz stimulation, $p > 0.5$ compared to 106.4 ± 4.7% for ACSF controls). Thus, ERK plays a role in Epac-mediated enhancement of LTP maintenance.

To firmly establish that 8-pCPT does not mediate these effects on LTP by activating PKA, I used KT5720 (1 μM), a PKA inhibitor. I found that application of KT5720 does not block the 8-pCPT-dependent facilitation of LTP maintenance. After 120 min, mean fEPSP slopes of 8-pCPT-treated slices were potentiated to 164.9 ± 11.0% compared to 107.9 ± 7.2% in ACSF control slices (**Figure 6.4D:** $p < 0.01$). These data indicate that 8-pCPT facilitates stability of LTP independently of PKA and supports the notion that this agonist selectively activates Epac.

e) LTP generated by pairing 8-pCPT with HFS occludes β-adrenergic receptor-dependent LTP

Activation of β-adrenergic receptors during one train of HFS also enhances the maintenance of LTP by initiating ERK signaling and new protein synthesis (Gelinas and Nguyen, 2005). To test the hypothesis that this form of neuromodulatory LTP is mechanistically similar to LTP generated by pairing 8-pCPT with HFS, I used an occlusion protocol. For this protocol, fEPSPs are reset to baseline slope values 30 min after one form of LTP is induced. A new baseline is then taken, followed by induction of

the second form of LTP. Co-application of the β-adrenergic agonist isoproterenol (ISO) and HFS following application of HFS alone did not inhibit the maintenance of β-adrenergic receptor-dependent LTP (**Figure 6.5A:** mean fEPSP slopes were potentiated to 145.3 ± 10.1% 120 min after ISO application). This result is consistent with the notion that decremental LTP induced by HFS alone recruits intracellular mechanisms distinct from those recruited by the long-lasting LTP generated by activation of β-adrenergic receptors during HFS. Thus, β-adrenergic receptor-dependent LTP can be maintained at synapses previously expressing decremental LTP.

However, co-application of ISO and HFS following application of 8-pCPT during HFS significantly occluded the maintenance of β-adrenergic receptor-dependent LTP (**Figure 6.5B:** mean fEPSP slopes were 105.2 ± 5.7% 120 min after ISO application, $p < 0.05$ compared to values from **Figure 6.5A**). This result suggests that activation of β-adrenergic receptors and application of 8-pCPT recruit similar mechanisms in order to facilitate long-lasting LTP. These mechanisms are 'saturated' by application of 8-pCPT, and subsequent activation of β-adrenergic receptors is unable to generate persistent LTP. Overall, these data indicate that activation of Epac or β-adrenergic receptors elicits mechanistically similar forms of long-lasting LTP.

f) 8-pCPT facilitates maintenance of chemically induced LTP

Although long-lasting LTP induced by activation of β-adrenergic receptors and Epac share similar mechanisms, activation of β-adrenergic receptors modulates the synaptic response to LFS, whereas Epac does not. To further investigate this difference, I used pharmacologic inhibition of phosphatases. Previous studies have shown that inhibition of phosphatase activity gates the induction of LTP (Blitzer et al., 1998; Brown

et al., 2000; Thomas et al., 1996). I found that inhibition of phosphatases with okadaic acid (1 μM) permitted the induction, but not the maintenance of LTP. LTP induced by applying LFS at 5 Hz for 3 min in okadaic acid-treated slices was decremental, with mean fEPSP slopes 101.5 ± 3.9% 120 min after LFS (**Figure 6.6A**). Similarly, LTP elicited by one train of HFS did not differ between okadaic acid-treated and control slices (**Figure 6.6C:** mean fEPSP slopes were 104.3 ± 4.8% in okadaic acid-treated slices and 109.2 ± 5.6% in ACSF-treated slices 120 min after HFS). These results suggest that phosphatase inhibition lowers the threshold for LTP induction without affecting LTP stability. As such, I examined whether application of 8-pCPT facilitates the maintenance of LTP induced by LFS during phophatase inhibition. Application of either ISO or 8-pCPT to okadaic acid-treated slices during LFS resulted in long-lasting LTP (**Figure 6.6B:** mean fEPSP slopes were 132.5 ± 5.8% and 131.6 ± 10.0% for ISO and 8-pCPT-treated slices, respectively, 120 min after LFS). Maintenance of this LTP was significantly enhanced compared to LTP generated by LFS alone applied to okadaic acid-treated slices (**Figure 6.6D:** $p < 0.01$). Overall, these results suggest that application of 8-pCPT facilitates the maintenance of LTP induced by chemical inhibition of phosphatases. They also lend support to the notion that induction and maintenance of LTP are served by mechanisms that can operate independently, and in parallel.

**4. Discussion**

I have examined the role of the cAMP-activated guanine exchange factor Epac in hippocampal synaptic plasticity. My data show that an Epac agonist enhances the maintenance of various forms of LTP in area CA1, without having significant effects on

basal synaptic transmission or initial LTP induction. Short-lasting, protein synthesis-independent LTP was converted into a stable, protein synthesis-dependent form of LTP by activation of Epac. I also demonstrated that the mechanism for this Epac-dependent LTP enhancement involves recruitment of ERK signaling.

Numerous signaling molecules contribute to the expression of LTP (Sanes and Lichtman, 1999). These molecules can mediate their effects by modulating various neuronal properties, including basal synaptic transmission and capacity for LTP induction or maintenance. I found that activation of Epac did not affect baseline synaptic responses or the initial magnitude of potentiation elicited. Similarly, Epac activation did not facilitate the induction of LTP by subthreshold synaptic stimulation (LFS). However, Epac activation significantly enhanced the maintenance of LTP generated by one train of HFS. Thus, pairing an Epac agonist with a stimulation protocol that is normally unable to elicit long-lasting potentiation of synaptic strength facilitated the expression of stable LTP. This potentiation was also stable for at least six hours *in vitro*. Importantly, the Epac-dependent enhancement of LTP was not blocked by pretreatment with a PKA inhibitor, confirming this effect is not mediated by concomitant PKA activation. Taken together, these results indicate that activation of Epac facilitates the stabilization of LTP.

The inability of Epac activation to facilitate LTP induction suggests that this signaling pathway may only be able to enhance LTP when sufficient synaptic stimulation is concurrently applied. My results demonstrate that Epac can also facilitate the maintenance of LTP induced by LFS when phosphatases are inhibited. Phosphatases can gate the induction of LTP, such that inhibition of phosphatases can substitute for activation of PKA (Blitzer et al., 1998). I found that pretreatment with the phosphatase

inhibitor okadaic acid permits induction of decremental LTP by LFS. Pairing LFS with Epac in slices pretreated with okadaic acid generates long-lasting LTP. Thus, Epac-dependent facilitation of LTP maintenance requires concomitant synaptic stimulation, in either electrical or chemical form.

Long-term changes in synaptic strength are distinguished from more transient forms of plasticity by their dependence on macromolecular synthesis (Frey et al., 1988; Frey et al., 1996; Kandel, 2001; Krug et al., 1984; Nguyen et al., 1994). I have shown here that the enhanced maintenance of LTP resulting from activation of Epac during LTP induction is protein-synthesis dependent, but independent of transcription. These results support the notion that translational activation of pre-existing dendritic mRNAs can sustain some forms of synaptic plasticity. For instance, β-adrenergic receptor-enhanced LTP (Gelinas and Nguyen, 2005), LTD induced by mGluR activation (Huber et al., 2001), and facilitation elicited by BDNF (Kang and Schuman, 1996) can be expressed in isolated CA1 dendrites that cannot receive newly transcribed mRNAs from the soma. Similarly, LTP generated by multiple trains of HFS can be stabilized by local protein synthesis for approximately three hours post-induction (Cracco et al., 2005; Tsokas et al., 2005). My present findings establish a putative cAMP-activated signaling pathway that can facilitate induction of translation-dependent, transcription-independent LTP. As such, Epac activation could occur downstream of Gs-protein coupled receptors or $Ca^{2+}$-activated adenylyl cyclases to participate in translational regulation of LTP. Indeed, over-expression of Epac potentiates the activation of ERK in response to stimulation of the Gs-protein coupled 5-$HT_{7A}$ receptors (Lin et al., 2003a). My current results further support this notion by demonstrating that the maintenance of β-adrenergic receptor-

dependent LTP is occluded by prior expression of Epac-enhanced LTP. Thus, these forms of LTP appear to share similar underlying mechanisms. However, the absence of a specific Epac antagonist prevents direct investigation of which receptors and/or stimulation patterns may recruit Epac to stabilize LTP.

My results also suggest a mechanism by which Epac activation elicits protein synthesis and persistent LTP. It is well established that the ERK-Mnk1-eIF4E pathway stimulates translation during generation of long-lasting plasticity and memory (Banko et al., 2006; Kelleher et al., 2004b; Schmitt et al., 2005). I showed that Epac-enhanced LTP requires ERK signaling. Furthermore, application of an Epac agonist tends to increase phosphorylation of ERK (data not shown here). Therefore, Epac may engage ERK signaling to stimulate translation initiation and generate long-lasting LTP.

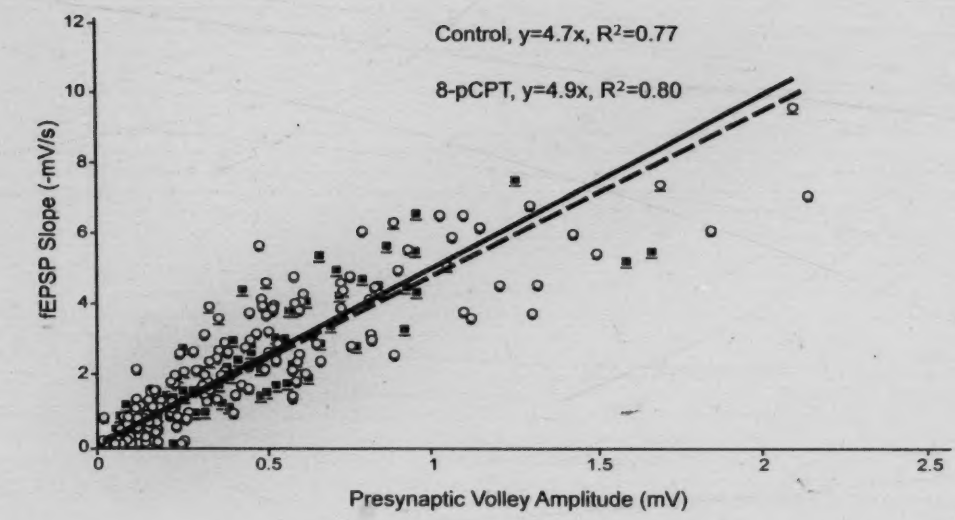
Epac is known to activate Rap in isolated cell lines (293T cells; CHO10001, CHO10248 cell lines), and Rap can interact with numerous signaling pathways, including the ERK cascade (de Rooij et al., 1998; Kawasaki et al., 1998). As such, Epac may activate ERK via Rap in area CA1 of the hippocampus. Indeed, the existence of an Epac-Rap-ERK pathway has been demonstrated in various systems (Keiper et al., 2004; Wang et al., 2006).

cAMP mediates diverse effects on synaptic plasticity, mostly through downstream activation of PKA. For instance, the cAMP-PKA pathway is strongly linked to transcriptional regulation of LTP (Impey et al., 1996; Impey et al., 1998). Interestingly, my results implicate the cAMP-Epac pathway in translational regulation of LTP. These distinct cAMP-dependent pathways may be involved in coordinating the regulation of transcription and translation during long-lasting LTP. It is also possible that different

plasticity-inducing signals selectively recruit Epac or PKA to generate variable downstream effects.

Epac-dependent facilitation of LTP maintenance in the hippocampus has potential physiological and behavioural ramifications. Hippocampal L-LTP is strongly correlated with explicit long-term memory (Abel et al., 1997; Bourtchuladze et al., 1994; Doyere and Laroche, 1992; Genoux et al., 2002). cAMP is also an important second messenger involved in both synaptic plasticity and memory (Abel et al., 1997; Frey et al., 1993; Nguyen and Woo, 2003). Thus, activation of Epac by cAMP is likely to be a functionally relevant occurrence in CA1 neurons. Epac could link cAMP to ERK and dendritic protein synthesis, allowing for rapid regulation of hippocampal long-term synaptic plasticity and modulation of memory. Epac expression levels are also altered in areas of the brain associated with Alzheimer's disease (McPhee et al., 2005), and its downstream targets are involved in a number of neurodegenerative and inflammatory conditions. My results establish a novel signaling pathway implicated in long-lasting hippocampal plasticity that may play an important physiological and therapeutic role in memory and neurocognitive disorders.

A.

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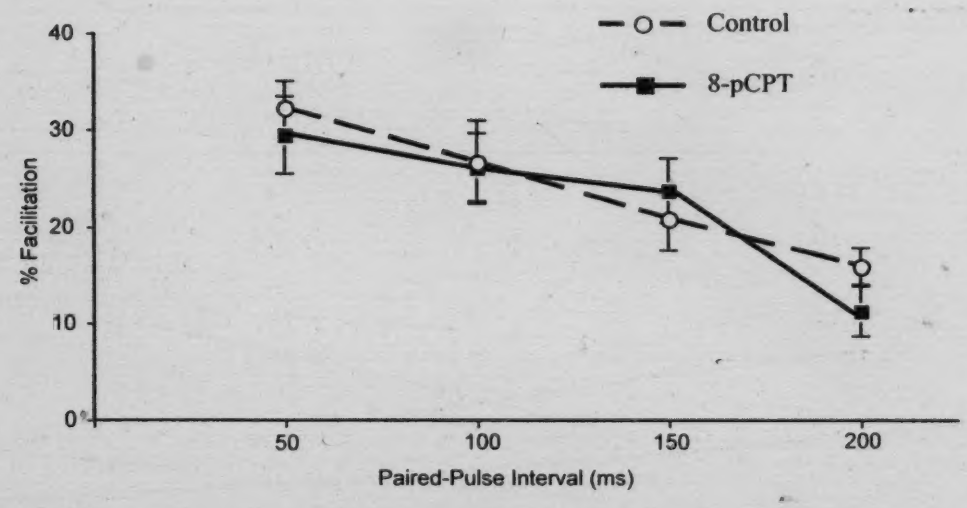


Figure 6.1: 8-pCPT does not alter neuronal excitability or presynaptic transmitter release capabilities. A) Input-output (I/O) curve slopes were not altered by application of 8-pCPT. Linear regressions were calculated for 8-pCPT-treated (n=12) and control slices (n=15). B) Paired pulse facilitation (PPF) was not altered by application of 8-pCPT. 8-pCPT-treated slices (n=13) exhibited facilitation similar to controls (n=16) at interpulse intervals of 50, 100, 150 and 200 ms.

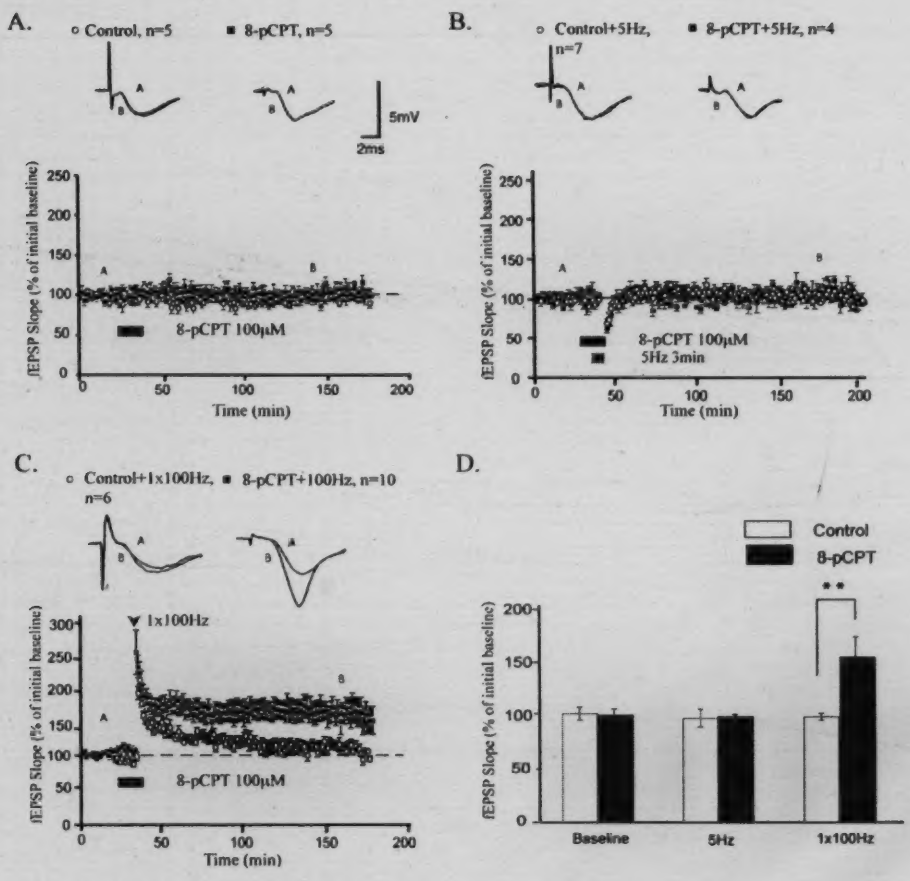


Figure 6.2: 8-pCPT enhances LTP maintenance, without affecting LTP induction or basal synaptic transmission. A) Application of 8-pCPT alone has no effect on basal synaptic transmission. B) Pairing 8-pCPT with 5 Hz stimulation has no long-term effect on synaptic strength. C) Pairing 8-pCPT with 1x100 Hz stimulation enhances maintenance, but not induction, of LTP relative to controls. D) Summary histogram for these experiments comparing levels of potentiation 120 min after LFS/HFS (**$p<0.01$). All sample fEPSP traces were sampled at time points "A" and "B" on graphs. Calibration bars: 5 mV, 2 ms.

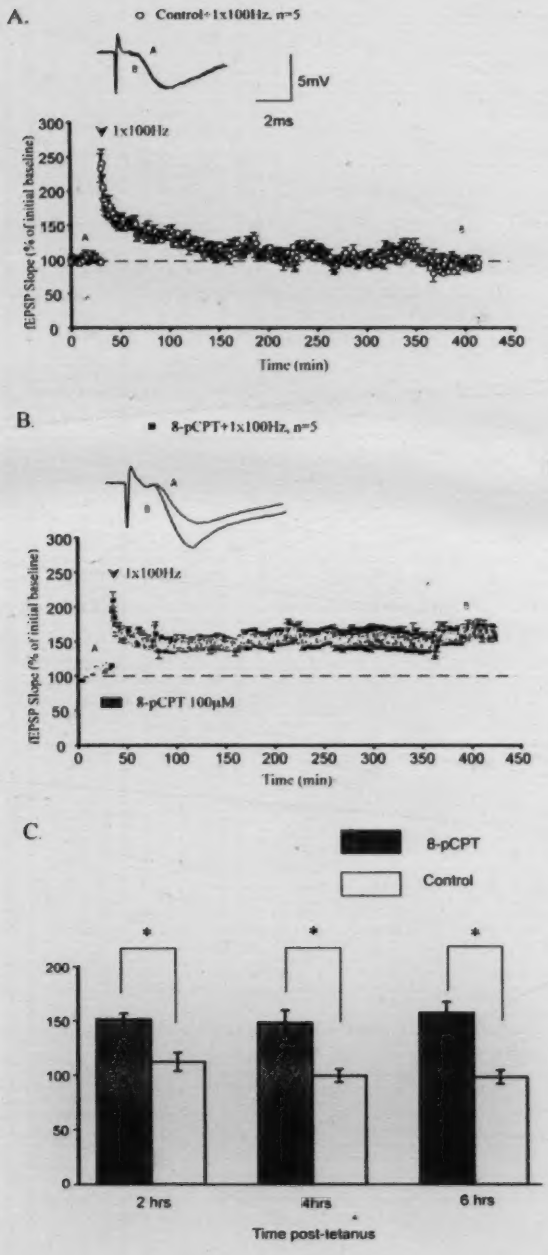


Figure 6.3: LTP generated by pairing 1x100 Hz stimulation with 8-pCPT is stable for at least six hours in vitro. A) 1x100 Hz stimulation alone elicits decremental LTP. B) Application of 8-pCPT during 1x100 Hz stimulation elicits stable LTP. C) Summary histogram for these experiments comparing levels of potentiation 2, 4, and 6 hours after HFS (*$p<0.05$). All sample traces were taken at time points "A" and "B" on graphs. Calibration bars: 5 mV, 2 ms.

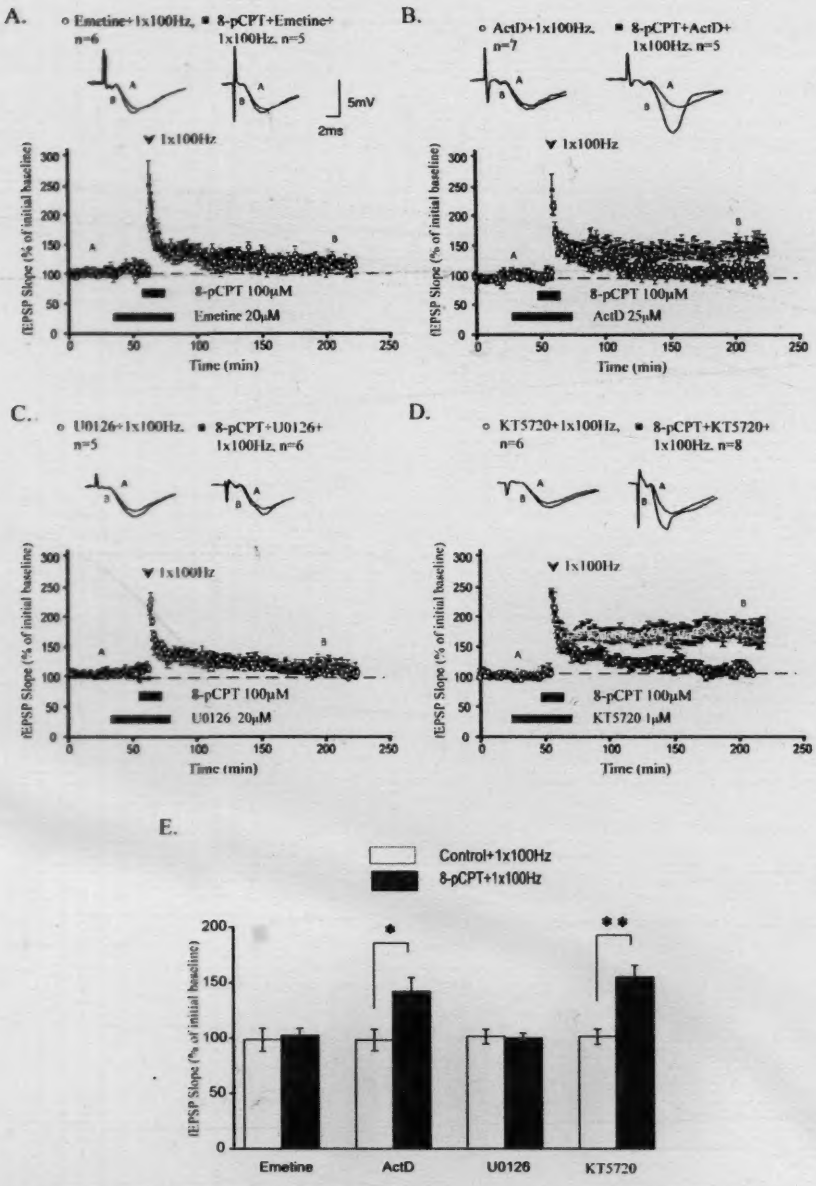


Figure 6.4: LTP elicited by pairing 1x100 Hz stimulation with 8-pCPT requires protein synthesis and ERK, but not transcription or PKA. A) Emetine, a translation inhibitor, blocked the enhancement of LTP by 8-pCPT. B) Actinomycin D, a transcription inhibitor, had no significant effect on LTP enhanced by 8-pCPT. C) U0126, an ERK inhibitor, also blocked the enhancement of LTP by 8-pCPT. D) KT5720, a PKA inhibitor, had no significant effect on LTP enhanced by 8-pCPT. E) Summary histogram for these experiments comparing levels of potentiation 120 min after HFS (**$p<0.01$, *$p<0.05$). All sample traces were taken at time points "A" and "B" on graphs. Calibration bars: 5 mV, 2 ms.

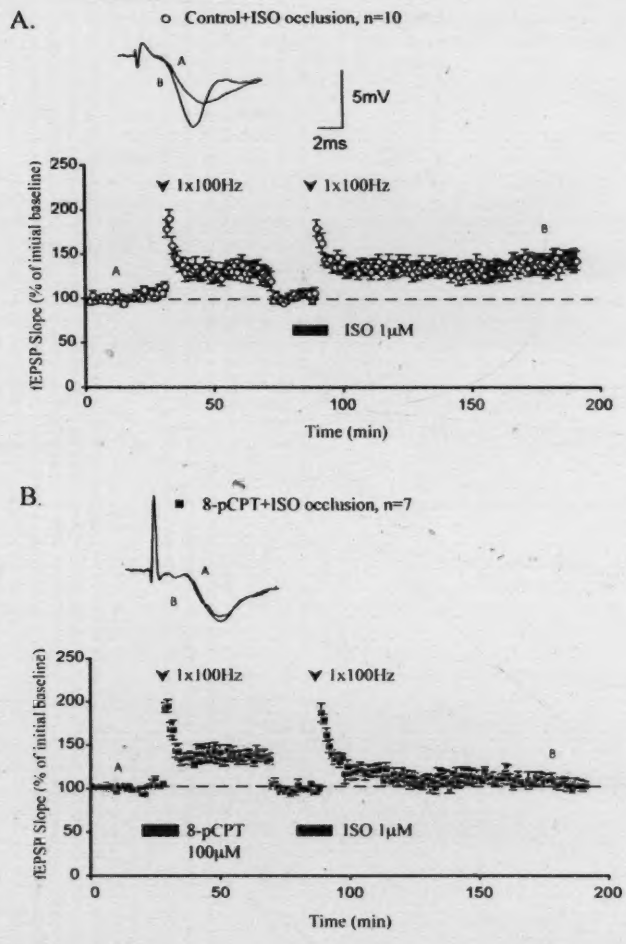


Figure 6.5: LTP generated by pairing 8-pCPT with 1x100 Hz occludes β-adrenergic receptor-dependent LTP. A) 1x100 Hz stimulation alone does not occlude subsequent maintenance of LTP generated by pairing application of ISO with 1x100 Hz stimulation. At 120 min after ISO+1x100 Hz, fEPSP slopes were potentiated significantly above the reset baseline. B) Pairing 8-pCPT with 1x100 Hz stimulation generates LTP that occludes subsequent maintenance of LTP generated by pairing application of ISO with 1x100 Hz stimulation. At 120 min after ISO+1x100 Hz, fEPSP slopes had decayed nearly to the reset baseline. All sample traces were taken at time points "A" and "B" on graphs. Calibration bars: 5 mV, 2 ms.

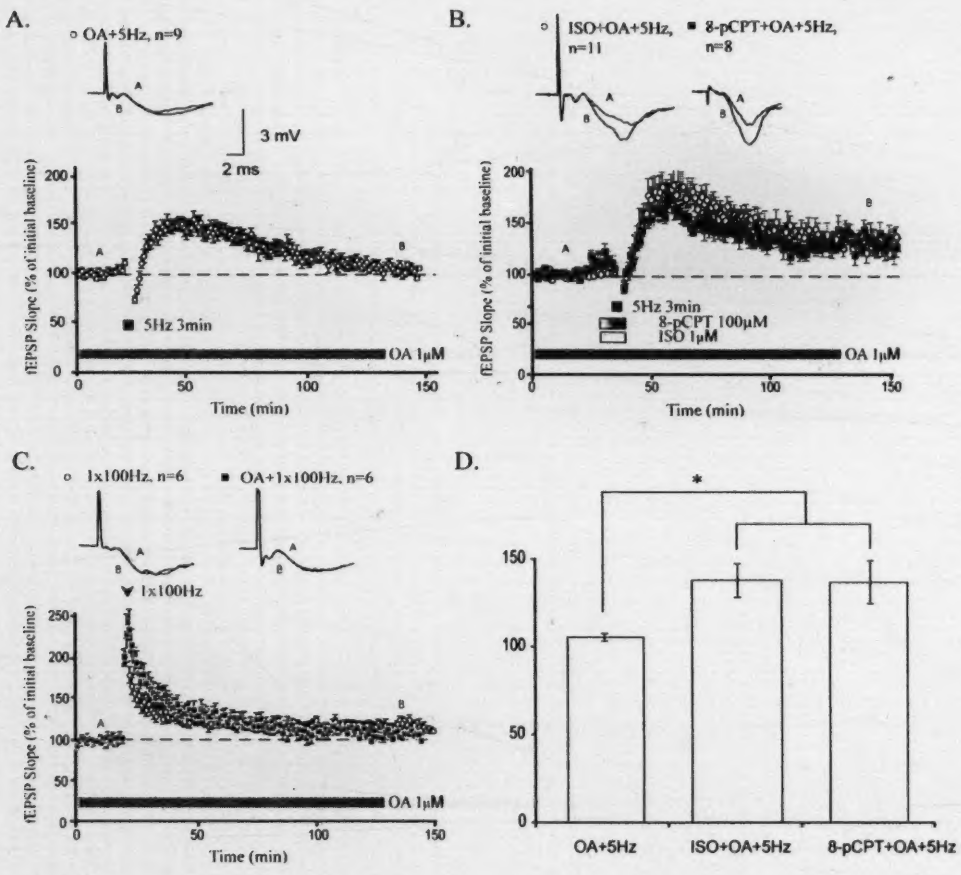


Figure 6.6: 8-pCPT enhances maintenance of chemically-induced LTP. A) Treatment with okadaic acid, a phosphatase inhibitor, permits the induction of decremental LTP in response to 5 Hz stimulation. B) Application of ISO, a β-adrenergic agonist, or 8-pCPT during 5 Hz stimulation enhances maintenance of LTP in okadaic acid-treated slices. C) Treatment with okadaic acid does not alter LTP generated by 1x100 Hz stimulation. D) Summary histogram for these experiments comparing levels of potentiation 120 min after HFS (*$p<0.05$). All sample traces were taken at time points "A" and "B" on graphs. Calibration bars: 5 mV, 2 ms.

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# CHAPTER VII

## General Discussion

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*Portions of this chapter have been submitted for publication:
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## 1. Noradrenergic Neuromodulation: Summary of Findings

Neuromodulatory systems of the brain regulate complex neural functions. Neurons from discrete nuclei in the brain stem can influence the entire CNS via the diffuse release of neuromodulators. Although much is known regarding the pharmacology and basic physiology of these neuroactive substances, the mechanisms by which they contribute to higher cognitive processes are unclear.

How the activity of individual neurons ultimately generates a unified percept that can be remembered and recalled is similarly unclear. However, significant progress has been made toward elucidating cellular correlates for the acquisition and retention of memories. Synaptic plasticity, measured as alterations in the strength of neural connections, fulfills many of the criteria for an information storage system (Bliss and Collingridge, 1993; Moser et al., 1998; Nathe and Frank, 2003). In the hippocampus, a brain region crucial for long-term memory, synaptic plasticity is well correlated with memory (Bourtchuladze et al., 1994; Doyere and Laroche, 1992; Genoux et al., 2002). Because the hippocampus is also densely innervated with axons of neuromodulatory neurons, it provides an excellent opportunity to investigate the involvement of neuromodulators in synaptic plasticity.

My research highlights the effects of noradrenergic modulation on synaptic plasticity in area CA1 of the hippocampus. I have identified several key properties of noradrenergic plasticity, and have elucidated signaling processes that underlie the long-term stability of this plasticity. Here, I will broadly summarize my research findings and speculate as to how the noradrenergic neuromodulatory system could interact with neuronal activity to influence both synaptic plasticity and memory.

a) Properties of Noradrenergic Synaptic Plasticity

The first objective of my thesis was to investigate the noradrenergic modulation of long-term synaptic plasticity. Although studies had previously addressed the effects of NA on neuronal excitability and LTP induction (Dunwiddie et al., 1992; Heginbotham and Dunwiddie, 1991; Katsuki et al., 1997), the consequences of noradrenergic receptor activation for LTP maintenance were mostly unknown. As demonstrated in Chapter III, application of NA to hippocampal slices *in vitro* transiently enhances synaptic strength in the absence of patterned electrical stimulation. When LFS that is normally incapable of inducing LTP is paired with NA, the result is long-lasting, stable LTP. These effects are mediated selectively by activation of β-, but not α-adrenergic receptors. As such, NA acts through β-adrenergic receptors to enhance the potency of subthreshold electrical stimuli and permit induction of LTP. Furthermore, activation of these receptors facilitates LTP maintenance, generating LTP that is non-decremental for at least two hours. The increased persistence of LTP following β-adrenergic receptor activation suggests an interesting parallel with enhanced LTM resulting from *in vivo* application of β-adrenergic agonists (Barros et al., 2001; Devauges and Sara, 1991; Izquierdo et al., 1998).

Consequently, in Chapter IV, I sought to determine the properties of β-adrenergic receptor-dependent LTP. To avoid concomitant activation of α-adrenergic receptors, I used a selective β-adrenergic receptor agonist, isoproterenol (ISO). Consistent with results obtained with NA application in Chapter III, application of ISO alone induced a small enhancement of synaptic transmission that faded soon after drug washout. Pairing ISO with LFS or a weak tetanus protocol also generated LTP that was stable for at least

six hours. These results indicated that activation of β-adrenergic receptors can facilitate maintenance of LTP generated by distinct electrical stimulation protocols, and further suggested a general role for these receptors in long-lasting plasticity.

This β-adrenergic receptor-mediated LTP was found to possess several key characteristics normally associated with L-LTP in the hippocampus. It was resistant to activity-induced reversal, or depotentiation, a commonly used indicator of LTP stability (Bashir and Collingridge, 1994; Woo and Nguyen, 2003; Young and Nguyen, 2005). The maintenance of β-adrenergic receptor-dependent LTP also required protein synthesis. *De novo* protein synthesis is a key characteristic of L-LTP and LTM (Davis and Squire, 1984; Deadwyler et al., 1987; Huang et al., 1996; Stanton and Sarvey, 1984). Although transcription is also required for several forms of L-LTP (Jones et al., 2001; Nguyen et al., 1994), pairing β-adrenergic receptor activation with subthreshold electrical stimulation elicited LTP that was not blocked by transcription inhibitors. Correspondingly, this form of LTP could be stably induced in isolated CA1 dendrites that cannot receive gene products from the cell body. β-adrenergic receptor-dependent LTP did not exhibit a late transcriptional component, remaining stable in the presence of transcription inhibitors for at least six hours *in vitro*. Activation of β-adrenergic receptors was therefore found to induce a late phase of LTP that was persistent, dependent on translation, but independent of transcription.

b) Molecular Mechanisms of β-adrenergic Synaptic Plasticity

The next objective of my thesis was to determine the signaling pathways and molecular mechanisms activated downstream of the β-adrenergic receptor during induction of long-lasting LTP. Because β-adrenergic receptor-dependent LTP was found

to engage dendritic protein synthesis (Chapter IV), I investigated the involvement of ERK and mTOR signaling cascades in this form of plasticity. ERK and mTOR are implicated in the translational regulation of both LTP and LTD, suggesting a conserved role in recruitment of protein synthesis at the synapse (Banko et al., 2006; Kelleher et al., 2004b; Tang et al., 2002). Consistent with this notion, ERK and mTOR were necessary for the maintenance, but not the induction, of LTP generated by pairing one train of HFS with application of a β-adrenergic agonist. Because β-adrenergic receptors couple to Gs-proteins and increase cAMP, I considered how ERK and mTOR were engaged downstream of these receptors. Given that PKA is a prime target of cAMP signaling, I examined the role of PKA in β-adrenergic receptor-dependent LTP. Surprisingly, I found that pharmacologic inhibition of PKA did not impair LTP elicited by pairing β-adrenergic receptor activation with one train of HFS, suggesting that β-adrenergic receptors can initiate signaling via pathways other than the classical cAMP-PKA pathway.

To firmly establish a link between ERK and mTOR signaling and recruitment of protein synthesis, I worked with Dr. Jessica Banko (previously at Baylor College of Medicine, currently at Vanderbilt Unversity) to assay biochemical indicators of protein synthesis. Translation of mRNA is strongly regulated at the level of translation initiation, and as such increased phosphorylation of key translation initiation factors is associated with accelerated rates of protein synthesis (Dever, 2002). Specifically, phosphorylation of the inhibitory binding protein 4E-BP enables formation of the translation initiation complex, and phosphorylation of translation initiation factor eIF4E enables efficient translation of mRNA (Beretta et al., 1996; Duncan et al., 2003; Gingras et al., 1998; Wang et al., 1998). In accordance with my electrophysiology results, I found that pairing

application of a β-adrenergic agonist with one train of HFS significantly increased phosphorylation of 4E-BP and eIF4E compared to application of either protocol alone. Immunohistochemistry further showed that these increases in phosphorylation occurred in CA1 dendrites (done in collaboration with Dr. Lingfei Hou at Baylor College of Medicine), supporting previous results indicating that this form of LTP is mediated by dendritic translation. Thus, induction of long-lasting β-adrenergic receptor-dependent LTP engages protein synthesis by activating translation initiation machinery. I also determined the roles of ERK and mTOR signaling cascades in mediating this activation. In line with other studies, I demonstrated that ERK stimulates the kinase Mnk1 to phosphorylate eIF4E, whereas mTOR is responsible for phosphorylation of 4E-BP (Banko et al., 2004; Banko et al., 2006; Tang et al., 2002). Because pharmacologic inhibition of mTOR does not prevent ERK or Mnk1 phosphorylation, and ERK inhibition does not impair 4E-BP phosphorylation, these signaling pathways operate independently and converge at regulation of eIF4E. This dual regulation of eIF4E suggests that it serves as a signal integration point, permitting protein synthesis only when ERK and mTOR are concurrently activated. I also found (in collaboration with Dr. Lingfei Hou at Baylor College of Medicine) that when 4E-BP2 is genetically absent, the activation of β-adrenergic receptors alone is sufficient to generate long-lasting LTP, lending support to the idea that control of eIF4E by 4E-BP is an important constraint on induction of stable synaptic plasticity. ERK- and mTOR-dependent regulation of protein synthesis therefore represents a mechanism for the enhanced maintenance of LTP elicited by activating β-adrenergic receptors during LTP induction.

I used PKA deficient transgenic mice to further investigate β-adrenergic receptor-dependent signaling during induction of long-lasting plasticity. LTP generated by pairing β-adrenergic receptor activation with one train of HFS was unimpaired in these mutant mice, consistent with the insensitivity of this LTP to pharmacologic inhibition of PKA. Furthermore, activating β-adrenergic receptors rescued deficits in LTP maintenance caused by genetic or pharmacologic PKA deficiency. These results suggest that β-adrenergic receptor signaling can operate in parallel with PKA signaling to facilitate the stability of LTP. However, genetic or pharmacologic PKA deficiency did impair induction of LTP generated by pairing β-adrenergic receptor activation with LFS, indicating that β-adrenergic receptor-dependent recruitment of signaling pathways is heavily dependent on concurrent patterns of synaptic activity. Taken together, these results provided insight into the signaling pathways engaged downstream of the β-adrenergic receptors and established a mechanism by which activation of these receptors could modulate the persistence of LTP.

c) PKA-Independent cAMP Signaling and Synaptic Plasticity

The recruitment of ERK and mTOR independently of PKA downstream of the β-adrenergic receptor suggested the existence of a PKA-independent cAMP signaling pathway (Chapter V). Such a signaling pathway could be generally involved in recruitment of dendritic protein synthesis and stability of LTP. I therefore investigated the effects of stimulating cAMP-GEFs (also known as Epac) on hippocampal synaptic plasticity. Because cAMP-dependent Epac activation stimulates Rap1, it can potentially initiate crosstalk with multiple intracellular signaling pathways (de Rooij et al., 1998). Using the Epac-specific agonist 8-pCPT, I found that Epac activation did not alter basal

synaptic properties or LTP induction. However, it selectively enhanced LTP maintenance, generating LTP that persisted for at least six hours *in vitro*. Furthermore, activation of Epac facilitated persistence of LTP induced by either one train of HFS, or by pharmacologic inhibition of phosphatases. Therefore, Epac-dependent enhancement of LTP maintenance does not specifically depend on the induction protocol employed. This facilitation of LTP maintenance also required protein synthesis and ERK signaling, but not transcription. Thus, Epac activation appears to recruit dendritic protein synthesis to stabilize LTP, similarly to activation of β-adrenergic receptors.

Given the apparent similarities between Epac-dependent LTP and β-adrenergic receptor-dependent LTP, I investigated whether they could occlude each other. Forms of LTP generally occlude one another if they share similar underlying mechanisms. In this case, inducing one form of LTP 'saturates' these mechanisms, and prevents subsequent establishment of the other form of LTP. I demonstrated that activation of Epac during one train of HFS elicits LTP that occludes the maintenance of β-adrenergic receptor-dependent LTP. These forms of LTP are therefore mechanistically similar, and Epac may indeed be activated downstream of the β-adrenergic receptor. However, this hypothesis cannot be properly examined until specific Epac antagonists are developed.

Overall, these studies establish an important role for β-adrenergic receptors in long-lasting synaptic plasticity. Furthermore, they reveal biochemical mechanisms that can locally stabilize LTP following β-adrenergic receptor activation. These mechanisms underscore the crucial influence of neuromodulators on synaptic plasticity, and provide insight into how these forms of synaptic stimulation could contribute to memory.

## 2. Establishment of Long-Lasting LTP – An Emerging Model

### a) Integration of Signaling

A hippocampal dendrite contains numerous types of postsynaptic receptors that enable it to respond to a wide variety of synaptic stimuli. Patterns of presynaptic activity are transmitted across the synapse via release of glutamate and subsequent activation of postsynaptic ionotropic glutamate receptors (Collingridge et al., 1988; Hablitz and Langmoen, 1982; Herron et al., 1985; Hestrin et al., 1990). Neuromodulatory afferents release neuroactive substances that stimulate a wide range of postsynaptic neuromodulatory receptors (Cooper et al., 2003). The question then arises: How are these diverse membrane receptor signals integrated within a dendrite to generate long-lasting synaptic plasticity? This issue is clouded by the bewildering array of molecules that can be activated downstream of these receptors to influence synaptic plasticity in the hippocampus (Sanes and Lichtman, 1999). As such, it is postulated that expression of long-lasting synaptic modifications results from the integration of many biochemical pathways, rather than the activity of one particular molecule or kinase (Kennedy et al., 2005). My research provides insight into how signals from neuromodulatory β-adrenergic receptors are combined with signals reflecting patterns of electrical activity to gate induction of long-lasting LTP. Furthermore, my results highlight the complex interplay between signaling pathways and underscore the differential involvement of these pathways in temporal phases of LTP. They also suggest a role for the recently discovered Epac pathway in hippocampal synaptic plasticity.

#### *i. Induction of β-adrenergic receptor-dependent LTP*

Consistent with previous studies, my results indicate that activation of β-adrenergic receptors facilitates LTP induction in area CA1 of the hippocampus (Chapter III; IV; (Thomas et al., 1996). Whereas LFS (5 Hz for 3 min) does not induce LTP, applying ISO during LFS generates robust LTP. The induction of this LTP requires PKA and ERK signaling, and can be mimicked by inhibition of phosphatases (Giovannini et al., 2001; Thomas et al., 1996; Winder et al., 1999). Furthermore, I demonstrated that either pharmacologic or genetic blockade of PKA activity inhibits establishment of LTP elicited by pairing ISO and LFS (Chapter V).

The signaling mechanisms that mediate LTP induction in response to pairing β-adrenergic receptor activation with LFS have been partially delineated. Activation of β-adrenergic receptors initiates PKA and ERK signaling cascades. PKA blocks phosphatase activity through phosphorylation of inhibitor-1, and promotes AMPA receptor function through phosphorylation of the GluR1 subunit. Dendritic potassium channel function is concurrently inhibited in an ERK-dependent manner to increase cellular excitability (Watanabe et al., 2002; Yuan et al., 2002). Synaptic stimulation at 5 Hz (theta frequency) causes action potential bursting in CA1 cells, and these action potentials can back-propagate into the dendrites. The ability of back-propagating action potentials to depolarize the neuron and activate NMDA receptors is enhanced when cellular excitability is increased (Morozov et al., 2003; Watanabe et al., 2002; Yuan et al., 2002). β-Adrenergic receptor-dependent modification of kinase and channel activity therefore permits robust NMDA receptor activation, and the subsequent induction of LTP in response to stimuli that would normally be subthreshold. Accordingly, pharmacologic inhibition of PKA or ERK decreases complex spike bursting of CA1 neurons and impairs

LTP induction (Winder et al., 1999). This interaction between signaling cascades generates a three-way coincidence detector, requiring synaptic activation, a back-propagating action potential, and β-adrenergic receptor-mediated inhibition of potassium channels to induce LTP (Sweatt, 2004). Thus, coincidence detection is an important method of signal integration, ensuring that LTP is induced only during appropriate patterns of electrical and neuromodulatory activity.

*ii. Maintenance of β-adrenergic receptor-dependent LTP*

Although PKA and ERK signaling pathways cooperate to elicit LTP in response to LFS, some evidence suggests that a different set of biochemical interactions is required to stably sustain this LTP. Pharmacologic inhibition of phosphatases allows induction of LTP in response to LFS, mimicking β-adrenergic receptor activation (Thomas et al., 1996). However, unlike β-adrenergic receptor-dependent LTP, this LTP was not long-lasting (Chapter VI). I also found that phosphatase inhibition was unable to facilitate the maintenance of LTP elicited by one train of tetanus. These results suggest that the signaling processes required for LTP induction are not necessarily the same as those required for LTP maintenance.

In an attempt to differentiate between signaling processes contributing to LTP induction and those contributing to LTP maintenance, I employed an alternate stimulation protocol. In Chapter IV, I established that activation of β-adrenergic receptors facilitates the maintenance of LTP generated by one train of tetanus, gating E-LTP into L-LTP. Because E-LTP elicited by one train of tetanus does not require PKA, ERK, or mTOR for its induction, this stimulation protocol provides the opportunity to specifically investigate signaling processes involved in LTP maintenance (Abel et al., 1997; Tang et al., 2002;

Winder et al., 1999). The increased stability of LTP resulting from concurrent β-adrenergic receptor activation was blocked by inhibitors of ERK and mTOR, but not PKA (Chapter V). Whereas PKA is recruited to induce β-adrenergic receptor-dependent LTP during LFS, it is not recruited for the maintenance of LTP during one train of HFS. Thus, different signaling pathways can be engaged for induction and maintenance of LTP depending on the concurrent patterns of synaptic activity. These signaling differences may reflect the finely tuned, yet poorly understood, ability of synapses to encode information contained in patterns of synaptic stimulation as differential modifications of synaptic strength.

Like induction of β-adrenergic receptor-dependent LTP, maintenance of this LTP involves coincidence detection. Signaling in ERK and mTOR pathways is necessary to engage local protein synthesis and generate long-lasting LTP downstream of the β-adrenergic receptor (Chapter V). Inhibition of either of these pathways results in impairment of β-adrenergic receptor-dependent LTP maintenance. At a biochemical level, this coincidence detection is accomplished by dual ERK- and mTOR-dependent regulation of the translation factor eIF4E. As such, protein synthesis is only recruited when ERK and mTOR are concurrently activated to a critical threshold. For instance, application of a β-adrenergic agonist modestly activates ERK and mTOR. Stimulation with one train of HFS similarly induces modest ERK and mTOR activation. However, pairing β-adrenergic receptor activation with one train of HFS additively increases the amount of ERK and mTOR signaling to generate long-lasting, protein synthesis-dependent LTP. Thus, signals from diverse postsynaptic receptors can be integrated at the level of translation initiation to cooperatively gate the persistence of LTP. This

process could represent a general mechanism by which activation of neuromodulatory receptors influences the physiological response to synaptic stimulation.

*iii. Divergent signaling*

β-Adrenergic receptors interact with Gs-proteins to stimulate adenylyl cyclase and increase cAMP (Minocherhomjee and Roufogalis, 1982; Raymond, 1995). Classically, cAMP then relieves the inhibition of PKA, activating PKA-dependent signaling and physiological responses (Beebe, 1994; Nguyen and Woo, 2003). However, the existence of β-adrenergic receptor-dependent, but PKA-independent, long-lasting LTP demonstrates that β-adrenergic receptors can activate diverse downstream targets. cAMP can also activate Rap1 via stimulation of cAMP-GEFs, also known as Epacs (de Rooij et al., 1998). The discovery of this signaling pathway raises the possibility that Gs-coupled receptors, including β-adrenergic receptors, can influence plasticity via activation of Epac. Indeed, several studies indicate that Epac is involved in Gs-coupled receptor-mediated effects (Johnson-Farley et al., 2005; Keiper et al., 2004; Lin et al., 2003a). My data provide evidence that Epac activation may be involved in β-adrenergic receptor-dependent plasticity. Firstly, application of an Epac agonist (8-pCPT) facilitates the maintenance of LTP when paired with one train of HFS. Like β-adrenergic receptor-mediated enhancement of LTP, this phenomenon requires protein synthesis and ERK activity, but not transcription. Epac-dependent long-lasting LTP also occludes β-adrenergic receptor-dependent LTP, suggesting that these forms of plasticity share similar underlying mechanisms.

The functional significance of separate cAMP-dependent signaling pathways that can influence hippocampal synaptic plasticity is speculative. The cAMP-PKA pathway is

strongly linked to transcriptional regulation of LTP (Impey et al., 1996; Impey et al., 1998), whereas my results suggest that the cAMP-Epac pathway may contribute to translational regulation of LTP. This divergent signaling downstream of cAMP could contribute to the conjoint transcriptional and translational regulation of long-lasting synaptic plasticity. It is possible that specific patterns of synaptic activity, or activation of certain receptors, preferentially activate either PKA or Epac. Conversely, crosstalk between these pathways could cooperatively regulate induction and/or maintenance of LTP. Subcellular compartmentalization is potentially a key determinant of signaling pathway activation near the postsynaptic membrane (Rich et al., 2000). As such, further investigation into the cellular localization of PKA and Epac should contribute to deciphering the information encoded in patterns of cAMP signaling.

b) Local Protein Synthesis and Synaptic Plasticity

My research contributes to a growing body of evidence suggesting that local protein synthesis can be engaged to establish long-lasting synaptic plasticity that is independent of somatic transcription. The functional implications of local protein synthesis are being actively investigated. Here I will discuss my findings in relation to current models of dendritic translation.

*De novo* protein synthesis is an established feature of L-LTP (Deadwyler et al., 1987; Huang et al., 1996; Stanton and Sarvey, 1984). mRNAs translated to generate these new proteins originate from either a pre-existing pool of mRNA in the dendrites, or from activity-dependent transcription in the nucleus. Local translational of pre-existing mRNAs has been observed in response to stimulation of NMDA receptors (Ouyang et al., 1999), application of an mGluR agonist (Job and Eberwine, 2001) and BDNF (Aakalu et

al., 2001). My results indicate that activation of β-adrenergic receptors or cAMP-GEFs (Epac) also initiates local protein synthesis (Chapter IV, V, VI). β-Adrenergic receptor-dependent LTP requires protein synthesis, but not transcription, and can be established in CA1 dendrites that have been isolated from their cell bodies. Similarly, Epac-dependent long-lasting LTP is protein synthesis-dependent, but transcription-independent (Chapter VI). These data strongly suggest that modulation of long-lasting plasticity by β-adrenergic receptor or Epac activation is exerted at a dendritic level. As more forms of synaptic stimulation are found to engage dendritic protein synthesis, the relationship between transcriptional and translational regulation of LTP must be closely examined. Although my results suggest that dendritic translation operates independently of transcription to establish long-lasting synaptic plasticity, it is likely that modifications at both the transcriptional and translational levels are important for consolidation of plasticity over an extended time period *in vivo*.

*i. Role of neuromodulators*

If synapses can acquire proteins necessary for long-lasting LTP via somatic or dendritic processes, what is the functional purpose of these distinct pathways? One possibility supported by my research findings is that local protein synthesis is engaged by activation of neuromodulatory receptors to rapidly initiate synaptic plasticity during weak synaptic stimulation. For instance, stimulation of synapses with LFS or weak tetanus does not engage protein synthesis-dependent LTP. However, pairing ISO with either of these stimulation protocols generates stable, protein synthesis-dependent LTP (Chapter IV). Activation of β-adrenergic receptors is important for the retention and retrieval of hippocampus-dependent memory (Ji et al., 2003a; Ji et al., 2003b; Murchison et al.,

2004), especially during periods of heightened emotional arousal (Cahill et al., 1994; McGaugh, 1989). As such, release of NA from noradrenergic afferents in the hippocampus could signal emotional relevance by enhancing the potency of synaptic activity that is normally subthreshold for induction of long-lasting plasticity. As seen in Chapter III, NA acts primarily through β-adrenergic receptors to facilitate LTP induction and maintenance. This modulation would allow state-dependent information from subcortical structures to influence processing of event-related information from the neocortex.

Similarly, combining subthreshold synaptic stimulation with application of a muscarinic agonist generates dendritic protein synthesis (Feig and Lipton, 1993). Given the putative role of acetylcholine in attentional processes (Sarter et al., 2005), acetylcholine-dependent local protein synthesis could signal attentional relevance. Overall, these studies indicate that certain neuromodulators can recruit dendritic translation to importantly influence synaptic plasticity. Although local protein synthesis has recently been demonstrated to underlie memory formation in Drosophila (Ashraf et al., 2006), the behavioural significance of protein synthesis initiated by neuromodulatory receptor activation in dendrites remains speculative.

*ii. Synaptic tagging*

Local protein synthesis may also contribute to a retrograde signal that elicits transcription in the nucleus (Klann and Dever, 2004; Martin and Kosik, 2002). In this manner, proteins rapidly translated in the dendrites could contribute to a sort of synaptic 'tag' for more persistent modification mediated by slower transcriptional processes. As discussed in Chapter I, the synaptic tagging hypothesis has been put forward to account

for the synapse specificity of LTP that requires gene products from the nucleus (for review, see (Martin and Kosik, 2002). In this model, transcriptional products are distributed throughout the neuron, but can only be utilized to stabilize LTP at previously active 'tagged' synapses. Interestingly, neuromodulatory systems are postulated to influence this tagging system by establishing an 'emotional tag' (Richter-Levin and Akirav, 2003). Heterosynaptic activation by neuromodulators could facilitate the establishment of L-LTP by reducing the threshold for tag formation, or initiating synthesis of plasticity-related proteins. My research findings provide support for this hypothesis. Activation of β-adrenergic receptors alone engages translation machinery, as demonstrated by increased phosphorylation and association of key translation initiation translation factors (Chapter V). Although these processes do not initiate persistent LTP, they could reduce the threshold for future L-LTP induction. Correspondingly, pairing β-adrenergic receptor activation with patterned electrical stimulation additively increases translation factor phosphorylation, and concomitantly elicits long-lasting, protein synthesis-dependent LTP (Chapter V). *In vivo*, activation of the amygdala can transform hippocampal E-LTP to L-LTP, and this process is dependent on muscarinic/noradrenergic neuromodulation (Frey et al., 2001). The β-adrenergic-dependent facilitation of long-lasting LTP could therefore represent a mechanism by which emotional information from the amygdala gates formation of L-LTP in the hippocampus.

My results also suggest a biochemical mechanism for this phenomenon. Mice that do not express the translational repressor 4E-BP2 demonstrate increased basal translation initiation (Banko et al., 2005). When β-adrenergic receptors are activated in

these mice, long-lasting LTP is elicited even in the absence of patterned electrical stimulation (Chapter V). This promiscuous induction of long-lasting LTP in response to diffuse neuromodulator release would eliminate the input specificity of LTP by circumventing the need for concurrent synaptic activity. As such, 4E-BP2 could play a critical role in regulating synaptic tagging and preserving specificity of information processing.

**3. Noradrenergic Synaptic Plasticity and Memory: Theoretical Implications**

My research findings have elucidated the role of NA in long-lasting synaptic plasticity in area CA1 of the hippocampus. Because considerable correlative evidence suggests that long-lasting forms of plasticity represent a cellular mechanism for information storage in the brain (Abel et al., 1997; Doyere and Laroche, 1992; Genoux et al., 2002; Martin and Morris, 2002), noradrenergic modulation of synaptic plasticity may contribute to memory processes. Indeed, NA likely plays a central role in memory processing in various brain regions (Brown and Silva, 2004; Harley, 2004; McGaugh, 2002).

The role of NA in LTM is supported by NA-dependent facilitation of long-lasting synaptic plasticity in hippocampal subregions. Consistent with studies of noradrenergic receptor subtypes involved in plasticity (Chapter III, (Katsuki et al., 1997), activation of β-adrenergic receptors accounts for the majority of NA-dependent memory effects (Devauges and Sara, 1991; Ji et al., 2003a; Ji et al., 2003b; Murchison et al., 2004). These receptors are implicated in long-term (LTM), rather than short-term (STM), memory processes. NA injected into area CA1 of the hippocampus selectively enhances LTM without altering STM (Izquierdo et al., 1998), an effect that parallels the increase in long-term stability of LTP resulting from pharmacologic application of NA during patterned electrical stimulation (Chapter III). Based on these temporal effects of β-adrenergic receptor activation, a role for the noradrenergic system in memory consolidation has been suggested (Izquierdo and

Medina, 1997; McGaugh, 2000). The ability of β-adrenergic receptor activation to recruit protein synthesis (Chapter IV, V), which is a cardinal feature of consolidated memory (Davis and Squire, 1984), supports this notion.

However, other studies propose that NA in the hippocampus is selectively involved in the retrieval, rather than acquisition or consolidation of memory (Murchison et al., 2004). Knockout mice that lack endogenous NA and adrenaline have been used to address this issue. These mice display β-adrenergic receptor-dependent deficits in long-term contextual fear memory that can be rescued by pre-testing, but not by pre-training, restoration of NA levels. Therefore, in the absence of NA, memories are acquired and consolidated, but cannot be retrieved. Noradrenergic activity in CA1 is implicated in this outcome, because patterns of neuronal activity in these NA deficient mice following exposure to a previously experienced, highly salient environment are normal in area CA3, but not area CA1 (Zhang et al., 2005). Zhang and colleagues therefore interpret the contextual memory retrieval deficits caused by lack of NA as resulting from the inability of area CA3 activation to be properly transmitted to area CA1. My data underscore the importance of NA in area CA1 in facilitating induction of synaptic plasticity, and also in rapidly stabilizing this plasticity. Because the cellular correlate for retrieval of memory is unknown, it is difficult to evaluate whether my results provide support for the memory retrieval hypothesis of NA function in area CA1. Some evidence suggests that both ERK and mTOR are required for memory retrieval (Chen et al., 2005). The ability of β-adrenergic receptors to recruit ERK and mTOR signaling cascades (Chapter V) could contribute to this memory process.

Noradrenergic activation in the amygdala can also facilitate hippocampus-dependent memory. Although the amygdala sends direct projections to the dentate gyrus (Ikegaya et al., 1996) and receives projections from the hippocampus in the ventral angular bundle (Maren and Fanselow, 1995), the mechanisms responsible for the memory-enhancing effects of amygdalar-hippocampal interactions are unknown. Some evidence suggests that theta activity contributes to this process. Neurons in the amygdala oscillate at theta

frequency (4-8Hz) during intense appetitive or aversive arousal (Pare and Collins, 2000). Similarly, high-amplitude theta activity is observed to modulate cellular responses in the hippocampus during arousal and locomotion (Bland et al., 1975; Buzsaki et al., 2003). Simultaneous recordings in the amygdala and hippocampal area CA1 reveal that rhythmically synchronized theta activity occurs between these two brain regions when animals are exposed to a conditioned fear stimulus (Seidenbecher et al., 2003). This oscillatory activity could promote interactions between the amygdala and hippocampus, potentially via thalamic pathways (Pare et al., 2002). Because activation of β-adrenergic receptors during theta frequency stimulation enhances LTP induction (Chapter III, IV; (Thomas et al., 1996), amygdala activation could potentiate the hippocampal response to NA. As such, oscillatory activity in the amygdala could facilitate the maintenance of long-lasting plasticity and memory.

Furthermore, the rescue of deficient L-LTP by β-adrenergic receptor activation has important ramifications for memory processes (Chapter V). R(AB) transgenic mice with deficits in PKA activity exhibit impaired maintenance of LTP generated by multiple trains of HFS. This impairment can be overcome by concomitant β-adrenergic receptor activation. Because multiple trains of HFS in area CA1 induce L-LTP that is independent of β-adrenergic receptors (Murchison et al., 2004; Sarvey et al., 1989; Swanson-Park et al., 1999) these results suggest that noradrenergic modulation can enhance mechanistically distinct forms of synaptic plasticity. Increasing noradrenergic activity similarly facilitates memory retention. Pharmacologic enhancement of NA release ameliorates memory deficits caused by aging or lesions of the basal forebrain (Haapalinna et al., 2000; M'Harzi et al., 1997). As such, drugs that increase NA in the brain have been proposed as treatments for memory disorders such as Alzheimer's disease (Chopin et al., 2002). The ability of β-adrenergic receptors to rescue deficiencies in maintenance of synaptic plasticity may underlie the therapeutic potential of these drugs.

## 4. Future Directions

### a) Further Questions

The discussion in the preceding paragraphs has identified some interesting questions that warrant further exploration. Firstly, how does activation of neuromodulatory receptors affect synaptic tagging? Stimulation of synapses with weak tetanus generates a synaptic tag that allows capture of protein products resulting from strong stimulation of nearby synapses. Although the nature of this tag is unknown, engagement or priming of dendritic translation machinery could contribute to tag formation (Martin and Kosik, 2002). Because I have shown that β-adrenergic receptor activation can also engage dendritic translation, it is possible that activation of these receptors can similarly generate a synaptic tag. Subsequent strong stimulation applied to independent synapses could provide protein products available for capture by these tagged synapses. It is also of interest to determine whether pairing β-adrenergic receptor activation with HFS is sufficient to generate protein products for capture at other weakly activated synapses. These mechanisms could extend the time window for information association during and after β-adrenergic receptor activation, and develop the role of synaptic tagging to include forms of neuromodulatory plasticity.

Next, how does regulation of translation initiation gate the establishment of bidirectional synaptic plasticity? My results indicate that activation of β-adrenergic receptors engages protein synthesis via ERK and mTOR signaling to elicit long-lasting LTP. However, ERK and mTOR signaling are also involved in the induction of long-lasting LTD downstream of metabotropic glutamate receptors (Banko et al., 2006; Gallagher et al., 2004; Hou and Klann, 2004). These conserved signaling mechanisms for regulation of translation initiation suggest that the direction of synaptic strength modification is determined by differential translation of mRNAs, or the existence of LTP/LTD-specific

tags. Identifying which mRNAs are translated in response to various forms of synaptic stimulation will provide important insight into this question.

Lastly, does endogenous Epac activation occur during hippocampal synaptic plasticity? I have demonstrated that application of an Epac agonist can selectively enhance the maintenance of LTP in area CA1 of the hippocampus. However, the absence of a specific Epac antagonist currently makes it difficult to investigate whether Epac is activated endogenously. Once such a pharmacologic agent exists, it will be interesting to determine whether Epac is ubiquitously activated in response to increases in cAMP, or if it is recruited by activation of specific receptors and patterns of synaptic activity. Given that Epac expression is altered in neural tissue that demonstrates pathology associated with Alzheimer's disease, this signaling pathway could also play a role in the pathology of memory (McPhee et al., 2005).

b) Methodological Advancements

Several methodological advancements could enhance our ability to investigate the questions highlighted in the preceding discussion. Firstly, the effects of NA on synaptic plasticity *in vitro* should be examined using *in vivo* systems. *In vivo* systems permit study of natural synaptic activity and neuromodulator release, creating a stronger link between plasticity and behaviour. For instance, the profile of receptors activated during endogenous release of NA is difficult to ascertain experimentally. This complicates *in vitro* studies that employ exogenous application of specific pharmacological agonists and antagonists. Cellular responses observed *in vitro* may not necessarily reflect cellular responses elicited by endogenous NA release in an *in vivo* system.

The subregions of the hippocampus also substantially differ in their neural responses to NA. *In vitro* studies that focus on plasticity effects in one subregion cannot reflect the processing capacities of the hippocampus in its entirety. As information is passed through the trisynaptic circuit of the hippocampus, NA could differentially affect computational properties of the subregions. More precise knowledge of circuit-level

interactions in this brain region is required to understand the potential contributions of noradrenergic receptors to plasticity and memory on a systems level. In this regard, novel neuroelectronic systems that couple neural tissue with a multi-transistor array on a silicon chip are being developed to permit high-resolution functional imaging of field potential activity. This technology allows the dynamics of neural activity to be correlated over large distances, providing insight into how synaptic activity spreads through hippocampal circuitry (Hutzler et al., 2006).

Understanding when and where protein synthesis is recruited during induction of long-lasting plasticity is limited by the lack of spatial and temporal control associated with conventional methods of pharmacologic protein synthesis inhibition. Bath application of protein synthesis inhibitors, such as anisomycin, generates cell-wide blockade of translation that is temporally regulated only by diffusion of the inhibitor. As such, precise determination of the spatiotemporal dynamics of plasticity-dependent translation is difficult. A new technique has been developed to overcome these difficulties. The creation of 'caged' anisomycin, which is activated only when exposed to a flash of UV light, permits precise, localized regulation of protein synthesis inhibition (Goard et al., 2005). Because this method can block protein synthesis in structures as small as a single dendritic spine, it would be highly useful to identify exactly when and where local protein synthesis is recruited to sustain β-adrenergic receptor-dependent LTP.

β-Adrenergic receptors engage the second messenger cAMP to mediate cellular responses. How cAMP differentially regulates the activities of numerous downstream targets is mostly unknown. The measurement of cAMP signals using real-time sensors provides an opportunity to examine the localization and kinetics of these signals (Karpen and Rich, 2004). Specifically, monitoring cAMP levels following activation of β-adrenergic receptors could shed light onto whether these receptors recruit signaling in specific subcellular compartments. Substantial evidence suggests that second messenger signals are generated in compartments near the plasma membrane, with limited diffusion between

compartments and the main cytosol (Rich et al., 2000). Signal compartmentalization could also be activity-dependent, explaining how β-adrenergic activation can recruit different intracellular signaling pathways in response to varied patterns of synaptic activity. Thus, use of these sensors could be an important step toward understanding how information from activation of neuromodulatory receptors is encoded in second messenger signals.

c) Conclusion

My thesis has concentrated on the role of NA in the maintenance of hippocampal synaptic plasticity, with a specific focus on the mechanisms underlying this form of neuromodulation. NA acts via β-adrenergic receptors in area CA1 of the hippocampus to generate long-lasting LTP that requires dendritic protein synthesis. Biochemically, β-adrenergic receptors can signal through ERK and mTOR pathways to engage translation initiation machinery and locally increase protein synthesis. Furthermore, the cAMP-dependent, but PKA-independent, Epac pathway may provide a link from β-adrenergic receptor-mediated increases in cAMP to recruitment of ERK (**Figure 7.1**). Although my findings provide insights into the influence of neuromodulatory receptors on synaptic plasticity, it is apparent that many issues remain to be investigated. Given the strong correlation between synaptic plasticity and memory in the mammalian brain, determining how NA influences information processing at cellular and behavioural levels is essential for understanding the physiology of memory. Such understanding may also reveal new strategies to improve treatments for human disorders of memory and cognition.

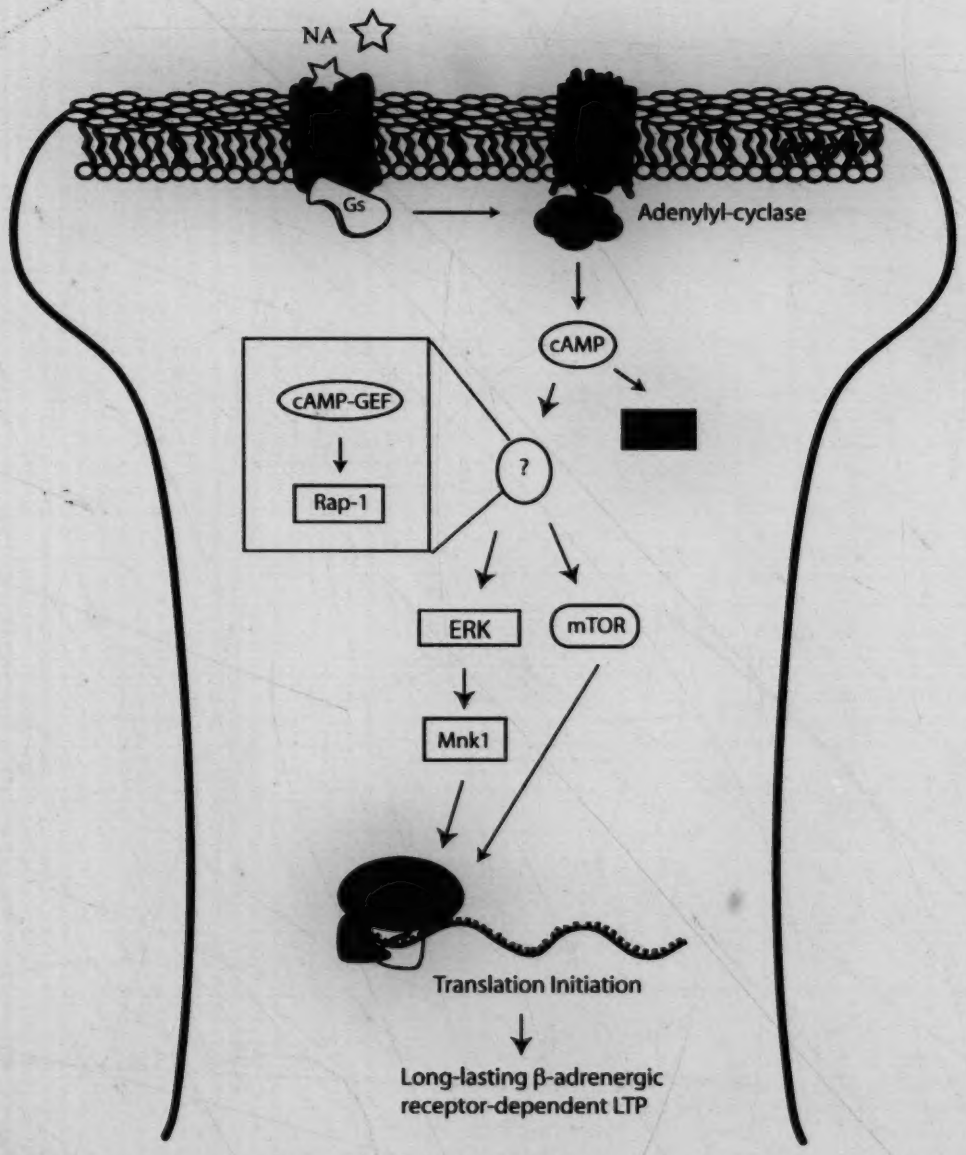


Figure 7.1: Potential signaling pathway linking β-adrenergic receptor activation to translation initiation and long-lasting LTP. β-Adrenergic receptors couple to adenylyl cyclase via Gs-proteins to increase cAMP. I found that β-adrenergic receptors recruit ERK and mTOR, but not PKA, signaling to initiate protein synthesis and generate long-lasting LTP. cAMP-GEF could provide a link between β-adrenergic receptor activation and downstream ERK and mTOR signaling. Established findings are shown in black and present findings are shown in blue.

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